



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : ZHI *et al.* Art Unit : 1625
Serial No. : 10/566,569 Examiner : Seaman, D. Margaret
Filed : August 21, 2006 Confirm. No.: 6058
Title : **6-CYCLOAMINO-2-QUINOLINONE DERIVATIVES AS ANDROGEN RECEPTOR MODULATOR COMPOUNDS**

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, **Lin Zhi**, declare as follows,

1) I am an inventor of the above-captioned application, which claims benefit of priority to U. S. provisional patent application Serial No. 60/497,125, filed 22 August 2003.

2) I obtained my B.S. and M.S. in chemistry from Beijing University in China. I obtained my Ph.D. in synthetic organic chemistry in 1990 from Emory University under the supervision of Prof. Albert Padwa. After postdoctoral training with Prof. Barry Trost in Stanford University, I joined Ligand Pharmaceuticals Inc. in 1992. I work in the area of small-molecule drug discovery targeted at intracellular/nuclear receptors. Currently, I hold the Senior Director position in Chemistry and Pharmaceutical Development at Ligand Pharmaceuticals Inc. I have over 60 publications and am an inventor on 60 issued US patents.

3) I have reviewed the Office Action, mailed February 2, 2010, in connection with the above-captioned application.

4) The above-captioned application provides selective androgen receptor modulator compounds. The compounds described in the above-captioned application interact with the androgen receptor (AR) to alter its activity.

5) I had each of Compounds 1-141, 144, 145, 148-177 and 179-189 tested for androgen receptor modulating activity using the cotransfection assay described in Example 55 using CV-1 cells. The test compounds were tested at a concentration of 10 μ M. AR agonist activity was compared to the activity of the known AR agonist dihydrotestosterone (DHT). The results are represented as percentage of DHT activity (DHT = 100% efficacy). AR antagonist activity was measured as the percentage of maximum inhibition of DHT at EC₅₀ concentration. For comparison, the inhibition of DHT at EC₅₀ concentration of the known AR antagonist bicalutamide is 100%. The results are shown in Table 1.

Table 1. AR modulating activity

Compound #	Agonist efficacy %	Antagonist efficacy %	Compound #	Agonist efficacy %	Antagonist efficacy %
101	112		145	125	
102	87		148	87	
103	117		149	26	
104	49		150	13	44
105	47		151	56	31
106	85		152	30	23
107	66		153	136	
108	147		154	126	
109	74		155	117	
110	138		156	92	
111	78		157	99	
112	81		158	26	
113	108		159	177	
114	117		160	58	
115	133		161	19	23
116		71	162	123	
117		59	163	30	21
118		78	164	130	
119	61		165	100	
120	107		166	90	
121	90		167	42	16
122	93		168	202	
123	105		169	60	33
124		42	170	113	
125	37		171	56	29
126	18	35	172	38	
127	30	36	173	95	
128	111		174	205	
129	120		175	100	
130	99		176	93	
131	69		177	35	
132	61	19	179	43	
133	41	41	180	141	
134	79		181	101	
135	113		182	163	
136		84	183	95	
137		80	184	138	
138	40	32	185	84	
139	96		186	35	
140	99		187	112	
141	95		188	68	
144	120		189	118	

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Declaration Pursuant to 37 C.F.R. §1.132

The results demonstrate that the exemplary compounds have AR modulating activity. Of the compounds tested, 78 compounds exhibit AR agonist activity. Of these 78 compounds, 36 compounds have AR agonist activity very similar to or better than the DHT control. The results demonstrate that 19 of the tested compounds exhibit AR antagonist activity.

Thus compounds as instantly claimed are AR modulators, including AR agonists, partial agonists and antagonists.

9) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

3/18/10
Date


Lin Zhi

Genotoxicity test results of representative compounds of Zhi *et al.*

Original test data can not be found.

COVANCE 21824-0-4098C									
TABLE 3: MUTAGENICITY ASSAY RESULTS - INDIVIDUAL PLATE COUNTS AND SUMMARY									
TEST ARTICLE ID: LG12678 (Lot No. LG12678-3355-10)									
EXPERIMENT ID: 21824-03									
DATE PLATED: 22-Nov-00									
DATE COUNTED: 27-Nov-00									
VEHICLE: DMSO									
PLATING ADJUST: 50 µL									
		REVERTANTS PER PLATE			MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION		BACK- GROUND LEVEL**		
DOSE/PLATE		TA1537			TA1537				
		1	2	3	MEAN	S.D.			
MICROSOMES: RAT LIVER									
VEHICLE CONTROL		8	9	8	9	2	1		
TEST ARTICLE									
	100 µg	10	26		23	4	1		
	333 µg	38	49		44	8	1		
	100 µg	92	94		93	1	1		
	333 µg	209	202		204	1	1		
	100 µg	491	557		524	47	1		
	333 µg	393	590		597	3	1sp		
	1000 µg	412	411		412	1	2sp		
POSITIVE CONTROL**		170	195		133	14	3		
MICROSOMES: NONE									
VEHICLE CONTROL		8	8	1	6	4	3		
TEST ARTICLE									
	100 µg	3	6		5	2	1		
	333 µg	7	3		5	3	1		
	100 µg	6	5		6	1	1		
	333 µg	8	7		8	1	1		
	100 µg	10	13		12	3	2		
	333 µg	0	0		0	0	4sp		
	1000 µg	0	0		0	0	5sp		
POSITIVE CONTROL***		1020	1019		1024	6	1		

** TA1537

2-aminofluorene

2.5 µg/plate

*** TA1537

100 µg/plate

2.0 µg/plate

*Black and White Evaluation Codes:

1 = normal

2 = slightly reduced

3 = moderately reduced

4 = extremely reduced

sp = slight precipitate

mp = moderate precipitate

hp = heavy precipitate

requires hand count

Compound 113 (LG0122940) negative:

Strain:	TA1537	Date 1:	3/6/2006
Compound:	LG0122940	2:	3/7/2006
S9 Activation:	wmwo S9	3:	3/12/2006
Cells seeded:	0		
(-) Control:	DMSO		

Concentration ug/ml	Test #		Revertants per Test		Average Revertants		Standard Deviation		Fold Increase		Fold Increase STOV	
			S9	noS9	S9	noS9	S9	noS9	S9	noS9	S9	noS9
(+) CTRL	2A	9A	S9	noS9	S9	noS9	S9	noS9	S9	noS9	S9	noS9
	1	1	37.0	192.0								
	2	2	62.0	192.0								
	3	3	48.0	192.0	49.00	192.00	12.5	0.0	29.40	15.57	23.03	#####
20.0												
	1	1	1.0	9.0								
	2	2	1.0	3.0								
	3	3	3.0	0.0	1.67	4.00	1.2	4.6	1.00	0.32	0.00	1.63
40.0												
	1	1	4.0	4.0								
	2	2	1.0	1.0								
	3	3	3.0	0.0	2.67	1.67	1.5	2.1	1.60	0.14	1.73	0.53
200.0												
	1	1	0.0	6.0								
	2	2	2.0	0.0								
	3	3	3.0	0.0	1.67	2.00	1.5	3.5	1.00	0.16	1.00	0.15
400.0												
	1	1	2.0	0.0								
	2	2	0.0	2.0								
	3	3	4.0	3.0	2.00	1.67	2.0	1.5	1.20	0.14	1.02	1.09
(-) Control:	DMSO											
	1	1	1.0	23.0								
	2	2	1.0	1.0								
	3	3	3.0	13.0	1.67	12.33	1.2	11.0	1.00	1.00	0.00	0.00

Exhibit C

Compound 114 (LGD2941) negative:

Genotoxicity: *In Vitro* Report Title: Bacterial Reverse Mutation Assay with an Independent Repeat Test Article: LGD2941
 Assay of LGD2941
 Test for Induction of: Reverse mutations in bacterial cells No. of Independent Assays: 2 Study No.: AB01TG.502001.BTL
 Strains: *S. typhimurium* and *E. coli* No. of Replicate Cultures: 3
 Metabolizing System: Aroclor-induced rat liver S9, 10% No. of Cells Analyzed/Culture: 0.4 to 1.2 X 10⁴
 Test Article DMSO Positive control vehicle: water (sodium azide) and DMSO (all others) GLP Compliance: Yes
 Vehicle:
 Treatment: Plate Incorporation for 48 hours Date of Treatment: 30-Nov-2004
 Cytotoxic Effects: at 1000 µg per plate with TA100 and TA1537 without S9
 Genotoxic Effects: None Experiment: B1

Metabolic Activation	Test Article	Dose Level (µg/plate)	TA1537	WP2	uvrA
None	DMSO	50 µL/plate	7 ± 1	14 ± 1	1
	LGD2941	3.3	6 ± 1	13 ± 2	2
		10	5 ± 3	12 ± 2	2
		33	5 ± 1	11 ± 2	2
		100	3 ± 2	15 ± 3	3
		333*	3 ± 2	15 ± 1	1
		1000*	4 ± 3	17 ± 3	3
	2-nitrofluorene	1.0			
	sodium azide	1.0			
	9-aminoacridine	75	1430 ± 25		
	methyl methanesulfonate	1000		152 ± 13	

Metabolic Activation	Test Article	Dose Level (µg/plate)	TA1537	WP2	uvrA
Rat liver S9	DMSO	50 µL/plate	6 ± 2	15 ± 2	2
	LGD2941	3.3	7 ± 2	16 ± 6	6
		10	4 ± 3	19 ± 2	2
		33	7 ± 1	15 ± 3	3
		100	7 ± 3	16 ± 1	1
		333*	5 ± 3	14 ± 2	2
		1000*	8 ± 2	18 ± 3	3
	2-aminoanthracene	1.0	89 ± 14		
		10		724 ± 18	

*Precipitating concentration

Exhibit D

Genotoxicity test results of 4-chloro analogs

Compound 130 (LG0123130) strongly positive:

Strain: 10-19-06 a
Compound: 10-19-06 b
S9
Activation: 11/9/2006
Cells seeded:
(-) Control:

Concentration µg/ml	Test #		Revertants per Test		Average Revertants		Standard Deviation		Fold Increase		Fold Increase STDV	
			S9	noS9	S9	noS9	S9	noS9	S9	noS9	S9	noS9
(+) CTRL	2AA	2NF										
	1	1	6.0	192.0								
	2	2	96.0	192.0								
	3	3	86.0	192.0	62.67	192.00	49.3	0.0	47.00	72.00	45.24	77.60
20.0												
	1	1	14.0	21.0								
	2	2	14.0	0.0								
	3	3	12.0	6.0	13.33	9.00	1.2	10.8	10.00	3.38	4.62	5.75
40.0												
	1	1	29.0	33.0								
	2	2	35.0	2.0								
	3	3	18.0	5.0	27.33	13.33	8.6	17.1	20.50	5.00	13.61	8.67
200.0												
	1	1	47.0	51.0								
	2	2	78.0	8.0								
	3	3	39.0	3.0	54.67	20.67	20.6	26.4	41.00	7.75	29.27	12.79
400.0												
	1	1	16.0	50.0								
	2	2	58.0	12.0								
	3	3	52.0	7.0	42.00	23.00	22.7	23.5	31.50	8.63	21.94	11.82
(-) Control: DMSO												
	1	1	1.0	2.0								
	2	2	1.0	1.0								
	3	3	2.0	5.0	1.33	2.67	0.6	2.1	1.00	1.00	0.00	0.00

Exhibit E

Compound 131 (LG0123131) strongly positive:

Strain: TA1537
Compound: LG0123131
S9
Activation: w/ & w/o S9
Cells seeded:
(-) Control:

10-19-06 a
10-19-06 b
11/9/2006

Concentration μ g/ml	Test #		Revertants per Test		Average Revertants		Standard Deviation		Fold Increase		Fold Increase STDV	
			S9	noS9	S9	noS9	S9	noS9	S9	noS9	S9	noS9
(+) CTRL	2AA	2NF										
	1	1	24.0	192.0								
	2	2	119.0	192.0								
	3	3	97.0	192.0	80.00	192.00	49.7	0.0	60.00	44.31	56.51	21.11
20.0												
	1	1	139.0	36.0								
	2	2	155.0	11.0								
	3	3	129.0	6.0	141.00	17.67	13.1	16.1	105.75	4.08	43.83	5.90
40.0												
	1	1	174.0	27.0								
	2	2	185.0	8.0								
	3	3	176.0	6.0	178.33	13.67	5.9	11.6	133.75	3.15	54.17	4.31
200.0												
	1	1	180.0	28.0								
	2	2	189.0	27.0								
	3	3	186.0	18.0	185.00	24.33	4.6	5.5	138.75	5.62	56.31	2.76
400.0												
	1	1	174.0	25.0								
	2	2	165.0	12.0								
	3	3	183.0	8.0	174.00	15.00	9.0	8.9	130.50	3.46	51.03	3.58
(-) Control: DMSO												
	1	1	2.0	3.0								
	2	2	1.0	7.0								
	3	3	1.0	3.0	1.33	4.33	0.6	2.3	1.00	1.00	0.00	0.00

Exhibit F

Compound 128 (LG0123128) negative:

7/2/2007

7/3/2007

Concentration µg/ml	Test #		Revertants per Test		Average Revertants		Standard Deviation		Fold Increase		Fold Increase STDV	
	<u>2AA</u>	<u>2NF</u>	<u>S9</u>	<u>No S9</u>	<u>S9</u>	<u>No S9</u>	<u>S9</u>	<u>No S9</u>	<u>S9</u>	<u>No S9</u>	<u>S9</u>	<u>No S9</u>
(+) CTRL	1	1	87.0	192.0								
	2	2	143.0	192.0								
					115.00	192.00	39.6	0.0	16.43	15.36	13.39	8.98
20.00	1	1	2.0	43.0								
	2	2	7.0	16.0								
					4.50	29.50	3.5	19.1	0.64	2.36	0.83	0.37
40.00	1	1	9.0	15.0								
	2	2	7.0	11.0								
					8.00	13.00	1.4	2.8	1.14	1.04	0.28	0.35
200.00	1	1	7.0	14.0								
	2	2	5.0	14.0								
					6.00	14.00	1.4	0.0	0.86	1.12	0.16	0.66
400.00	1	1	7.0	12.0								
	2	2	10.0	10.0								
					8.50	11.00	2.1	1.4	1.21	0.88	0.86	0.38
(-) Control: DMSO	1	1	9.0	17.0								
	2	2	5.0	8.0								
					7.00	12.50	2.8	6.4	1.00	1.00	0.00	0.00

Exhibit G

Compound 129 (LG0123129) negative:

Strain:	TA1537	1/30/2006
Compound:	LG0123129	1/31/2006
S9		
Activation:	wnwo S9	2/20/2006
Cells seeded:	0	
(-) Control:	DMSO	

Concentration ug/ml	Test #		Revertants per Test		Average Revertants		Standard Deviation		Fold Increase		Fold Increase STDV	
(+) CTRL	1	1	100.0	192.0								
	2	2	97.0	58.0								
	3	3	3.0	3.0	66.67	84.33	55.2	97.2	15.38	18.07	25.69	34.71
20.00	1	1	2.0	3.0								
	2	2	3.0	10.0								
	3	3	3.0	3.0	2.67	5.33	0.6	4.0	0.62	1.14	0.36	0.14
40.00	1	1	5.0	6.0								
	2	2	6.0	9.0								
	3	3	3.0	3.0	4.67	6.00	1.5	3.0	1.08	1.29	0.95	0.54
200.00	1	1	4.0	1.0								
	2	2	7.0	8.0								
	3	3	3.0	3.0	4.67	4.00	2.1	3.6	1.08	0.86	0.62	0.38
400.00	1	1	5.0	4.0								
	2	2	3.0	8.0								
	3	3	3.0	3.0	3.67	5.00	1.2	2.6	0.85	1.07	1.09	0.19
(-) Control:	DMSO											
	1	1	2.0	3.0								
	2	2	8.0	8.0								
	3	3	3.0	3.0	4.33	4.67	3.2	2.9	1.00	1.00	0.00	0.00



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Examiner : Seaman, D. Margaret
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ATTACHMENTS

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Spironolactone Directly Inhibits Proliferation of Cultured Human Facial Sebocytes and Acts Antagonistically to Testosterone and 5 α -Dihydrotestosterone *In Vitro*

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Spironolactone produces antiacne effects and has recently been shown to inhibit 5 α -dihydrotestosterone (5 α -DHT) receptors in human sebaceous glands. We applied spironolactone alone and combined with testosterone and 5 α -DHT to investigate its effects on the proliferation of human sebocyte cultures derived from facial skin. Secondary human facial sebocytes in 96-well culture plates were treated for 10 d by a single or combined application of testosterone (10^{-8} – 10^{-5} M), 5 α -DHT (10^{-8} – 10^{-5} M), and spironolactone (10^{-12} – 10^{-7} M) in serum-free basal medium. Cell proliferation was assessed in six wells using a fluorometric assay. Testosterone and 5 α -DHT significantly stimulated sebocyte

proliferation in a dose-dependent manner, the effect being strongest with 5 α -DHT. Spironolactone, on the other hand, caused a dose-dependent inhibition (25% and 50% at 10^{-9} and 10^{-7} M, respectively). Combined treatment of human facial sebocytes with spironolactone and testosterone or 5 α -DHT resulted in a lower proliferation than with androgens alone. The fact that spironolactone directly and dose dependently inhibits the proliferation of cultured human facial sebocytes and acts antagonistically to testosterone and 5 α -DHT at the cellular level is indicative of a receptor-mediated effect. *J Invest Dermatol* 100:660–662, 1993

Spironolactone is an aldosterone antagonist that has been clinically used as an antihypertensive and diuretic drug. The fact that gynecomastia is a common side effect of this compound reflects its antiandrogenic property [1,2]. Spironolactone has also been successfully used for treatment of acne [3,4], hirsutism [5], and androgenic alopecia [6]. The following mechanisms have been thought to explain its action: 1) interference with steroid synthesis by blockage of cytochrome P 450 enzymes in the testes and adrenal glands [7,8]; 2) reduction of 5 α -reductase activity [9]; and 3) peripheral action by a competitive decrease in 5 α -dihydrotestosterone (5 α -DHT) activity at the receptor level, as has been demonstrated both in experimental animals and in humans [10,11].

Our aim was to better elucidate the effects of spironolactone on the sebaceous glands at the cellular level by investigating its direct influence on the proliferation of cultured human facial sebocytes *in vitro* as well as its possible modification of testosterone and 5 α -DHT activity in this connection.

MATERIALS AND METHODS

Human Sebocyte Cultures Human sebaceous glands were isolated from facial skin and seeded on monolayer 3T3 cells, as previ-

ously described [12]. Primary sebocyte cultures were derived from the periphery of the gland lobules and were maintained to confluence before subcultivation. All experiments were performed using secondary sebocyte cultures, which have been demonstrated to consist of cells undergoing sebocytic differentiation [13,14].

Treatment with Testosterone, 5 α -DHT, and Spironolactone Human sebocytes were seeded in 96-well culture plates (Falcon, Jersey, NJ) at a concentration of 10^4 cells/well and were left to attach for 2 d at 37°C with 5% CO $_2$ in culture medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (Gibco, Berlin, Germany) supplemented with 8% fetal calf serum (Seromed, Berlin, Germany), 2% human serum, 10 ng/ml epidermal growth factor (Sigma, Deisenhofen, Germany), 10^{-9} M cholera toxin (Calbiochem, Frankfurt, Germany), 3.4 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (all from Seromed). The medium was then aspirated, and serum-free keratinocyte basal medium (KBM) (Clonetics, San Diego, CA) without additives supplemented with testosterone (10^{-8} – 10^{-5} M) (Sigma), 5 α -DHT (10^{-8} – 10^{-5} M) (Sigma), or spironolactone (10^{-12} – 10^{-7} M) (Searle Yakuhin, Osaka, Japan) or their combination was added to six wells at each concentration. The compounds were added to the medium as a 0.2% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) solution. KBM with 0.2% DMSO was concomitantly added to another six wells serving as controls. The plates were incubated at 37°C for 10 d before evaluation. KBM with and without compounds was changed every 2 d.

Cell Proliferation Cell numbers of untreated human sebocytes in 96-well culture plates were assessed over 9 d by counting single-cell suspensions in Neubauer chambers and were compared with the absolute fluorescence units (AFU) of parallel wells obtained using the 4-methylumbelliferyl heptanoate (MUH)–fluorescence assay [15,16]. This assay is based on the hydrolysis of the fluorogenic

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Abbreviations:

AFU: absolute fluorescence units

5 α -DHT: 5 α -dihydrotestosterone

DMSO: dimethyl sulfoxide

KBM: serum-free keratinocyte basal medium

MUH: 4-methylumbelliferyl heptanoate

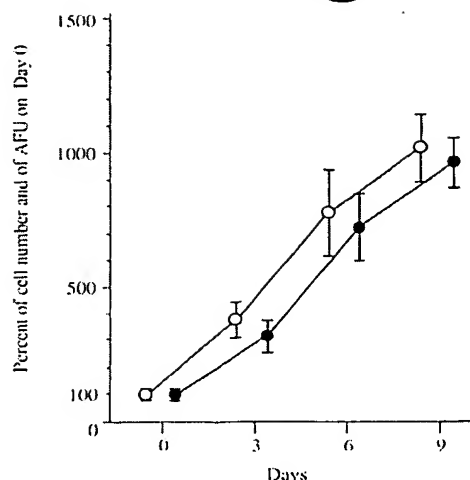


Figure 1. Comparison of cell numbers of proliferating human sebocytes in culture (open circles) and of AFU in parallel cultures obtained using the MUH-fluorescence assay (closed circles). Values are mean \pm SD of six wells and are presented as percent of cell number and percent of AFU on the day of assessment compared with day 0.

substrate MUH by esterases of proliferating cells. Briefly, a stock solution of 10 mg/ml MUH (Serva, Heidelberg, Germany) was prepared in DMSO and kept frozen at -20°C until use. On the day of assessment, KBM was removed, and the cells were washed twice with phosphate-buffered saline without Ca^{++} and Mg^{++} (pH 7.2) (Seromed). The MUH stock solution was diluted in phosphate-buffered saline up to 100 $\mu\text{g}/\text{ml}$, and 100 μl of the final solution was added to each well. The plates were then incubated for 30 min at 37°C and read automatically on a Titertek Fluoroscan II (Flow, Meckenheim, Germany). The MUH-fluorescence assay was used to assess the effects of androgens and spironolactone on sebocyte proliferation. The results are given as AFU using 355-nm excitation and 460-nm emission filters.

Statistical Evaluation Each value represents the mean of six wells \pm 1 SD. Statistical significance was assessed by Student *t* test. Mean differences were considered significant at $p < 0.05$.

RESULTS

Sebocyte proliferation was highly correlated with the results obtained using the MUH-fluorescence assay (Fig 1). Spironolactone inhibited the proliferation of cultured human sebocytes derived from sebaceous glands of the facial skin in a significant and dose-dependent manner. The inhibitory effect was 25% at 10^{-9} M and 50% at 10^{-7} M (10^{-9} , 10^{-8} , and 10^{-7} M; $p < 0.01$) (Fig 2).

Testosterone stimulated the proliferation of cultured human facial sebocytes, the effect being 25% at 10^{-7} – 10^{-6} M and 50% at 10^{-5} M (10^{-6} and 10^{-5} M; $p < 0.01$) (Fig 3a). 5α -DHT markedly enhanced the proliferation of cultured human facial sebocytes in a dose-dependent manner: 25% at 10^{-8} M and 50% at 10^{-7} M (for all concentrations tested $p < 0.01$) (Fig 3b).

Spironolactone significantly inhibited the stimulatory effect of testosterone on the proliferation of cultured human facial sebocytes *in vitro*. Inhibitory effects were 3–5% at 10^{-9} M, 14–22% at 10^{-8} M, and 22–36% at 10^{-7} M of spironolactone when added together with different testosterone concentrations to human facial sebocyte cultures (spironolactone 10^{-8} M plus testosterone 10^{-8} M, $p < 0.05$; spironolactone 10^{-7} M plus testosterone 10^{-7} , 10^{-6} , and 10^{-5} M, $p < 0.05$; spironolactone 10^{-7} M plus testosterone 10^{-8} M, $p < 0.01$) (Fig 3a).

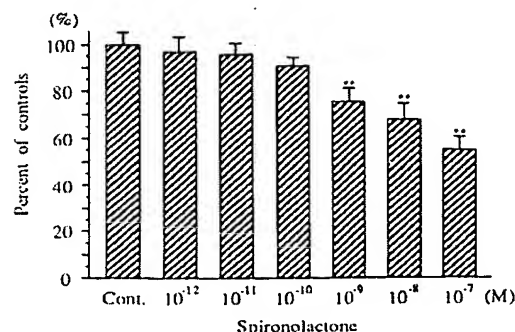


Figure 2. Effect of spironolactone on the proliferation of cultured human sebocytes derived from sebaceous glands of facial skin, as assessed by the MUH-fluorescence assay. Values are mean \pm SD of six wells and are presented as percent of controls (Cont.). ** $p < 0.01$, compared with controls.

In a similar way, the stimulatory effect of 5α -DHT on the proliferation of cultured human facial sebocytes was dose dependently reduced by spironolactone. Inhibitory effects were 14–20% at 10^{-9} M, 19–30% at 10^{-8} M, and 22–45% at 10^{-7} M of spironolactone when added together with different 5α -DHT concentrations to the proliferating cells: (spironolactone 10^{-9} M plus 5α -DHT 10^{-8} M, $p < 0.05$; spironolactone 10^{-8} M plus all 5α -DHT concentrations tested, $p < 0.05$; spironolactone 10^{-7} M plus 5α -DHT 10^{-8} M, $p < 0.05$; spironolactone 10^{-7} M plus 5α -DHT 10^{-7} , 10^{-6} , and 10^{-5} M, $p < 0.01$) (Fig 3b).

DISCUSSION

Androgens cause hyperactivity of sebaceous glands, with increased sebum secretion [17]. The resulting seborrhea promotes the formation of acne lesions [18]. The major circulating androgen, testosterone, is intracellularly converted in the skin to 5α -DHT by the enzyme 5α -reductase [19,20]. The androgenic effect of 5α -DHT is probably mediated by its binding to androgen receptors. Androgen receptors have been demonstrated in human sebaceous glands [21,22]. Therefore, hypersecretion of androgens and increased testosterone metabolism to 5α -DHT are closely correlated to the pathogenesis of acne [23–27].

In this study, the proliferation of cultured human facial sebocytes was shown to be significantly stimulated by testosterone and 5α -DHT but markedly inhibited in a dose-dependent manner by spironolactone and testosterone or 5α -DHT simultaneously administered. These observations indicate that spironolactone antagonizes testosterone and 5α -DHT activity, inhibiting their stimulatory influence on the proliferation of human facial sebocytes *in vitro*. Evidence suggests that such an effect may be due to inhibition of 5α -reductase reduction of testosterone to 5α -DHT [9] and partial or total blockage of 5α -DHT binding to its receptor [10,11]. In addition, increasing doses of spironolactone produced a corresponding proliferation inhibition indicative of a receptor-monitored effect. Thus, spironolactone may antagonize 5α -DHT by binding to its receptor or by modifying the structure of its receptor. It seems likely that the ligand–receptor interaction is disturbed.

An inhibitory effect of spironolactone on 5α -DHT-stimulated proliferation of human sebocytes derived from femoral skin *in vitro* was previously observed by our study group [28]; however, sebocytes may respond differently to androgens, depending on the location of the sebaceous glands [29]. The results of our study also confirm the inhibitory effect of spironolactone on testosterone- and 5α -DHT-stimulated proliferation of sebocytes derived from facial skin.

In conclusion, spironolactone may produce its antiacne effect by

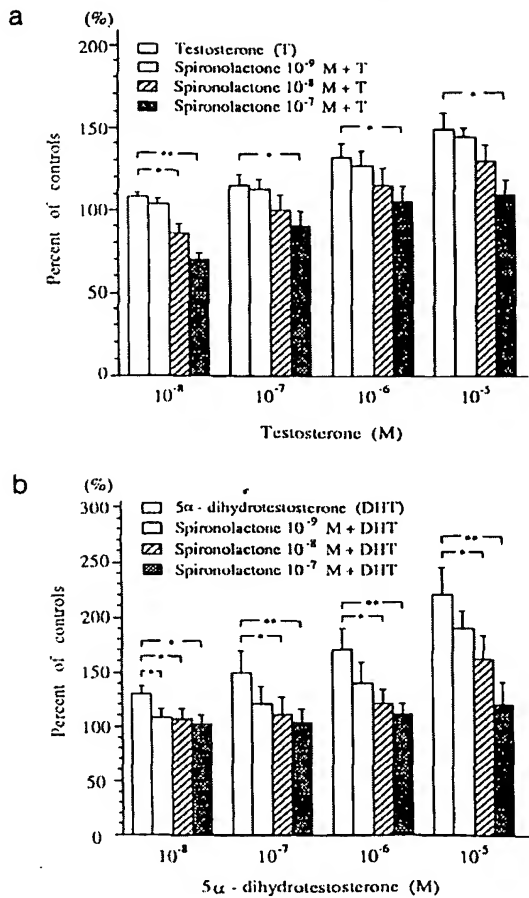


Figure 3. Effect of spironolactone on the stimulatory influence of (a) testosterone and (b) 5 α -DHT on the proliferation of cultured human sebocytes derived from sebaceous glands of facial skin, as assessed by the MUH-fluorescence assay. Values are mean \pm SD of six wells and are presented as percent of controls. * $p < 0.05$, ** $p < 0.01$, compared with testosterone- and 5 α -DHT-treated controls, respectively.

directly inhibiting the proliferation of human sebocytes and by acting antagonistically on the stimulation of the sebaceous gland by testosterone and, above all, by 5 α -DHT at the cellular level. Both effects seem to be receptor mediated.

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Expert Opinion

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2. Goals of therapy
3. Available compounds
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5. Conclusions
6. Expert opinion

Monthly Focus: Endocrine & Metabolic

Male hormonal contraceptives

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As the world human population continues to explode, the need for effective, safe and convenient contraceptive methods escalates. Historically, women have borne the brunt of responsibility for contraception and family planning. Except for the condom, there are no easily reversible, male-based contraceptive options. Recent surveys have confirmed that the majority of men and women would consider using a hormonal male contraceptive if a safe, effective and convenient formulation were available. Investigators have sought to develop a male hormonal contraceptive based on the observation that spermatogenesis depends on stimulation by gonadotropins, follicle-stimulating hormone (FSH) and luteinising hormone (LH). Testosterone (T) and other hormones such as progestins suppress circulating gonadotropins and spermatogenesis and have been studied as potential male contraceptives. Results from two large, multi-centre trials demonstrated that high-dosage T conferred an overall contraceptive efficacy comparable to female oral contraceptives. This regimen was also fully reversible after discontinuation. However, this regimen was not universally effective and involved weekly im. injections that could be painful and inconvenient. In addition, the high dosage of T suppressed serum high-density lipoprotein (HDL) cholesterol levels, an effect that might increase atherogenesis. Investigators have attempted to develop a hormonal regimen that did not cause androgenic suppression of HDL cholesterol and that was uniformly effective by suppressing spermatogenesis to zero in all men. Studies of combination regimens of lower-dosage T and a progestin or a gonadotropin-releasing hormone analogue have demonstrated greater suppression of spermatogenesis than the WHO trials of high-dosage T but most of these regimens cause modest weight gain and suppression of serum HDL cholesterol levels. Overall, the data suggest that we are close to developing effective male hormonal contraceptives. The focus is now on developing effective oral regimens that could be safely taken daily or long-acting depot formulations of a male hormonal contraception that could be conveniently injected every 3 - 6 months. In this article, we shall review the exciting new developments in male hormonal contraception.

Keywords: androgen, gonadotropin-releasing hormone antagonists, gonadotropins, male contraception, progestin, spermatogenesis, testosterone

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1. Introduction

The world's population continues to increase rapidly. As a result of the burgeoning population growth, there are disastrous environmental consequences and oppressive poverty in many parts of the world. In addition, inadequate contraception leads to high rates of unwanted pregnancies that lead to high rates of abortions, infanticide or unwanted children who suffer from abuse and neglect. In the past 50 years, many new contraceptive options for women have been developed. However, the only male options remain condoms and vasectomy. Both of these options have significant drawbacks. Condoms may decrease the pleasurable sensations of sexual intercourse,

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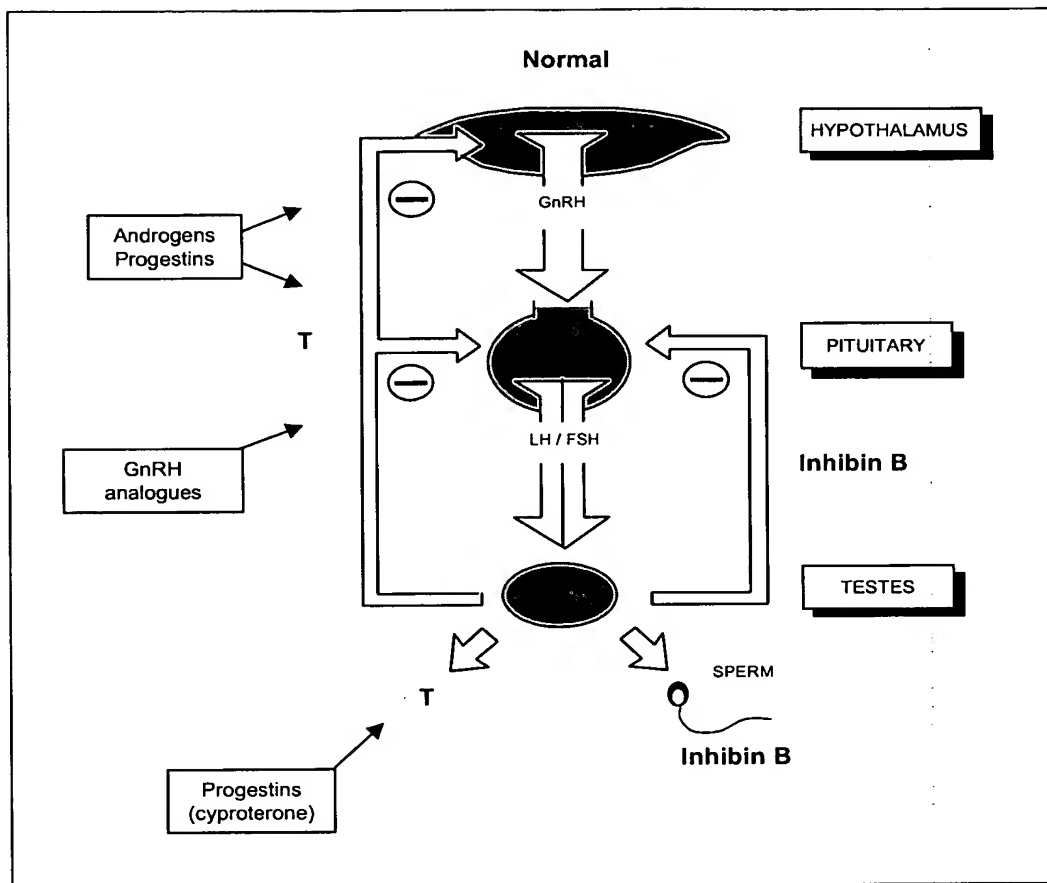


Figure 1. Mechanisms of action of contraceptive agents on normal male gonadal axis. The normal axis functions as a feedback loop with T and its metabolites inhibiting FSH and LH and inhibin B inhibiting FSH secretion. GnRH analogues inhibit GnRH action on the pituitary, so that FSH and LH secretion are suppressed. Androgens and progestins inhibit the hypothalamus and pituitary and some progestins might also have direct inhibitory effects on spermatogenesis in the testes.
FSH: Follicle-stimulating hormone; GnRH: Gonadotropin releasing hormone; LH: Luteinising hormone; T: Testosterone.

and they are associated with a high long-term failure rate. For practical purposes, vasectomy must be considered a permanent, irreversible form of contraception. Thus, there is an urgent need for safe, convenient, effective and reversible male contraceptive options.

Normal spermatogenesis depends on stimulation of the testes by circulating FSH and LH that is produced in the pituitary. Potential targets for pharmacological disruption of spermatogenesis include the hypothalamus (where gonadotropin-stimulating hormone [GnRH] is produced), the pituitary or directly in the testes (Figure 1). Male hormonal contraceptive regimens that are currently under investigation

act primarily by suppressing circulating gonadotropins. Administration of exogenous T alone suppresses circulating gonadotropins and spermatogenesis. However, exogenous T must be administered at supraphysiological dosages to effectively suppress spermatogenesis. Other agents, such as GnRH analogues and/or progestins, act synergistically with exogenous T and permit lower, more physiological, dosages of T to suppress gonadotropins and spermatogenesis.

2. Goals of therapy

The ideal hormonal contraceptive would reduce sperm concentrations of all men to azoospermia, the complete absence of sperm in the ejaculate. However, it might not be necessary to induce azoospermia to achieve excellent contraceptive effectiveness. Good evidence from clinical trials of exogenous T alone suggest that suppression of sperm concentrations from the normal range (20 - 200 million per ml) to oligospermia (> 0 but ≤ 3 million) or oligoazoospermia (≤ 3 million sperm/ml) confers excellent contraceptive efficacy (pregnancy rate of < 5 per 100 person-years). Suppression to severe oligoazoospermia (≤ 1 million/ml) is even more effective than oligoazoospermia (pregnancy rate of < 2 per 100 person-years). Therefore, experts regard suppression to oligoazoospermia or severe oligoazoospermia as good secondary goals in clinical trials of male hormonal contraceptives.

3. Available compounds

3.1 Androgens

3.1.1 *T enanthate alone as a contraceptive*

Exogenous im. administration of T enanthate alone has been shown to be an effective male contraceptive in two studies sponsored by the WHO. The first study enrolled 271 men from ten centres in seven countries [1]. These men were administered T enanthate (200 mg) im. weekly during a 6-month induction phase. The 157 (58%) who became azoospermic were enrolled in a 12-month efficacy phase when no other contraception was used while continuing weekly im. T enanthate. Only one pregnancy occurred among the 119 couples during the 1486 cumulative months of follow-up of the couples who completed the efficacy phase. The pregnancy rate of 0.8 per 100 person-years (the Pearl index) compares favourably with the efficacy of the oestrogen/progestin birth control pill for women.

After establishing the contraceptive efficacy of T-induced azoospermia in normal young men, a second multi-centre WHO trial was conducted to determine the contraceptive effects of both T-induced azoospermia and near-azoospermia [2]. A total of 399 men from nine different countries (123 from Asian countries and 276 from non-Asian countries) were recruited. Fertility had been demonstrated in 86% of the male subjects and 77% had demonstrated fertility with their partners at the time of the study. A total of 357 (89%) completed the 6-month induction phase of T enanthate (200 mg im.) weekly and 98% of the men who completed the induction phase had sperm counts that suppressed below the study's target threshold of oligospermia (≤ 3.0 million/ml). Of the men from Asian and non-Asian countries, 90 and 70% became azoospermic, respectively, after the induction period. During the 12-month efficacy period of using only T enanthate for contraception, there were no pregnancies fathered by the men who became azoospermic. Of the men who did not become azoospermic but whose sperm counts suppressed to ≤ 3 mil-

lion/ml, the rate of resultant pregnancies was reduced to 8.1 per 100 person-years. Of the men who did not become azoospermic but whose sperm counts suppressed to ≤ 1 million, the pregnancy rate was 5.1 per 100 person-years. The combined pregnancy rate for all men who suppressed to $0 \leq 3$ million/ml (98% of the men suppressed to ≤ 3 million/ml) was 1.4 per 100 person-years. The overall contraceptive efficacy, including those subjects who failed to suppress to oligoazoospermia (0 - 3 million/ml), was better than 95%; comparable to or better than the reported contraceptive efficacy of the female birth control pill or barrier methods such as the condom.

The two WHO trials demonstrated that androgen-based contraceptives could be used as safe, effective and reversible male contraceptives. The major side effects of the T enanthate regimen used in the two WHO trials were modest weight gain (2.5 - 3.0 kg) and a significant decline in HDL cholesterol (10 - 15%) levels. These effects could potentially increase long-term risk of atherosclerosis. It is likely that the increased weight is due to increased lean body mass without changing fat mass [3]. In addition, in the second WHO trial, low density lipoprotein (LDL) cholesterol levels decreased significantly and this decrease in the atherogenic LDL levels might offset the any harm due to decreased serum HDL cholesterol levels.

3.1.2 *Longer-acting formulations of androgens*

Although the WHO trials showed that a T-based male contraceptive is effective, a major barrier to widespread use of this regimen is the requirement for weekly im. injections. In addition, chronic administration of T enanthate (200 mg) im. weekly produces peak, mid-cycle and trough serum total and free T levels that are supraphysiological [4]. Therefore, longer-acting formulations of im. T are being studied. The half-life of unmodified T is very short because the liver rapidly metabolises it. Testosterone that is esterified to enanthate or cypionate and injected intramuscularly in an oil vehicle (e.g., sterile sesame oil) has a significantly longer half-life. T cypionate or enanthate must be administered im. every 1 - 2 weeks, but other T esters, such as T undecanoate, T buciclate and T decanoate, may be given at longer intervals (Figure 2).

T undecanoate is an unsaturated, aliphatic fatty acid ester of testosterone. Intramuscular T undecanoate (in sterile tea or sesame seed oil) has been shown to maintain serum T levels in the normal range for at least 6 - 8 weeks in hypogonadal men [5,6]. Intramuscular T undecanoate has been studied in several human male contraceptive trials. In a small pilot study, healthy young Chinese men were randomised to 500 mg or 1000 mg of T undecanoate im. monthly. In total, 11/12 in the 500 mg group and all 12 in the 1000 mg group suppressed to azoospermia [7]. The subject in the 500 mg group who did not become azoospermic suppressed to severe oligoazoospermia (≤ 1 million/ml). Serum testosterone levels rose, but remained in the normal range and serum HDL cholesterol levels were unaffected in both groups. However, in a

Male hormonal contraceptives

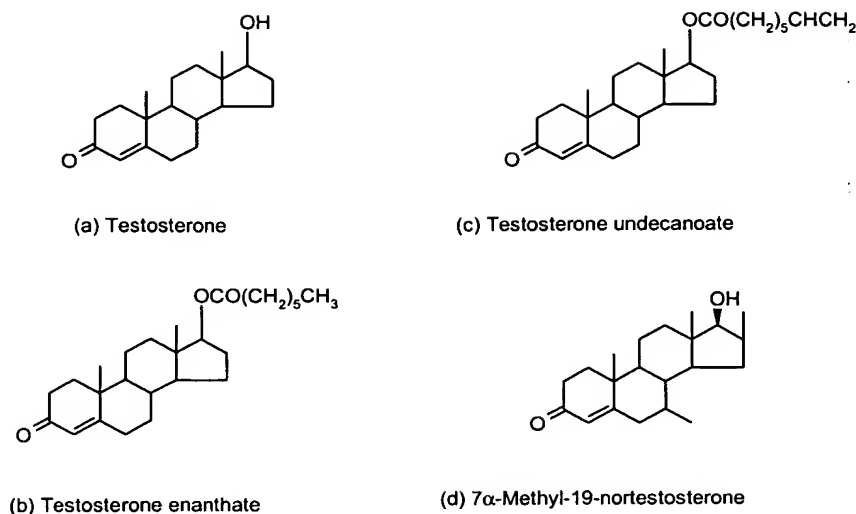


Figure 2. Androgens used in contraceptive research. (a) testosterone (b) testosterone enanthate (c) testosterone undecanoate (d) 7α-methyl-19-nortestosterone (MENT).

study of German men, only 8/14 men became azoospermic after receiving 1000 mg of im. T undecanoate every 6 weeks for 24 weeks [8]. In contrast to the Chinese study, T undecanoate caused a significant decrease in serum HDL cholesterol levels in the German men (~10%). Overall, T undecanoate is at least as effective as weekly injections of T enanthate for suppressing spermatogenesis in Asian and non-Asian men, and T undecanoate has superior pharmacokinetic properties that would permit longer intervals (up to at least 6 weeks) between injections.

Other long-acting T formulations have been developed that might be useful but they have not been tested extensively in male hormonal contraceptive regimens. T buclate is a long-acting T ester prepared in an aqueous suspension of finely milled crystals. When administered im. as a single dose of 600 or 100 mg to hypogonadal men, T buclate raised and maintained serum T levels in the low-normal range for up to 16 - 20 weeks [9]. In a pilot study, a single dosage of 1200 mg of T buclate induced azoospermia in 3/8 eugonadal young men while maintaining serum T levels in the normal range [10]. Finally, Organon NV is investigating T decanoate, an ester with a similar pharmacokinetic profile as T undecanoate, as a part of a male contraceptive regimen.

Testosterone may be encapsulated in biodegradable polylactide-glycolide microspheres for administration as a depot injection that releases T with first order absorption kinetics. In hypogonadal men, im. injection of a second-generation formulation of T microspheres maintained serum T levels in the normal range for 10 - 11 weeks [11]. We have recently found that a third-generation T microcapsule formulation may be administered sc. with similar pharmacokinetics. A long-acting sc. T microcapsule formulation might be an effective

and convenient preparation for a male hormonal contraceptive regimen because sc. injections tend to be less painful than im. injections.

Testosterone may also be packaged as pellets of crystalline T with a diameter of 4.5 mm and a length of 6 mm (100 mg) or 12 mm (200 mg). When implanted surgically under abdominal skin, 600 - 1200 mg of T pellets provide relatively stable physiological serum T levels for up to 4 - 6 months [12]. Implantation of 1200 mg of T pellets is comparable to 200 mg of weekly im. T enanthate for inducing azoospermia [13]. A recent efficacy trial of sc. implantation of T pellets confirmed that 800 - 1200 mg every 3 months suppressed sperm counts comparably to that reported for high-dosage T enanthate used in the WHO trials. There were no pregnancies during this small study and side effects were minimal [14]. The T pellets are not quite optimal because implantation is a minor surgical procedure that requires special training and because they occasionally extrude (~8 - 10%).

3.1.3 Non-injectable formulations of androgens: oral and transdermal T preparations

There are several non-injectable formulations of T now available for use in male contraceptive regimens. These options include oral T undecanoate, transdermal T patch systems and transdermal T gel. However, few contraceptive trials have been conducted with these agents and no studies have been conducted using them as single agents for male contraception.

Unmodified T is metabolised rapidly by the liver and it has a short half-life when ingested orally. Alkylated (at the 17α position) T has a longer half-life. These alkylated androgens may cause serious hepatotoxicity and are too dangerous to use clinically for the treatment of hypogonadism or in contracep-

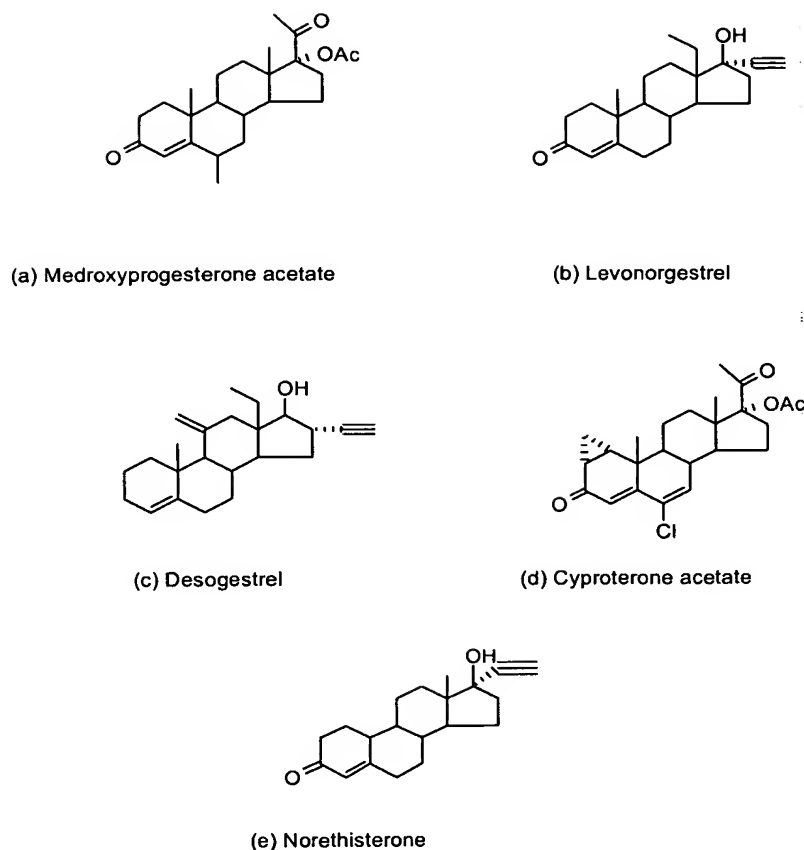


Figure 3. Progestins used in male contraceptive research. (a) medroxyprogesterone acetate, (b) levonorgestrel, (c) desogestrel, (d) cyproterone acetate and (e) norethisterone.

tion regimens. T undecanoate contains a medium chain ester that allows the compound to be absorbed by the lymphatic system, thus bypassing a first-pass hepatic effect [15]. Although it does not cause hepatotoxicity, oral T undecanoate still has a relatively short half-life and it must be administered 2 - 4 times daily. Oral T undecanoate plus cyproterone acetate shows promise as a potential oral male contraceptive pill (discussed under progestin section below) [16].

There are scrotal and non-scrotal T patch systems that raise serum testosterone levels to the normal or low-normal range when applied daily to hypogonadal men [17]. Although both patch systems can be self-applied and are painless, the scrotal patch systems require frequent scrotal shaving, the non-scrotal systems often cause a rash and neither patch system provides enough testosterone to adequately suppress circulating gonadotropins and spermatogenesis enough for effective male contraception [18]. The United States FDA has recently approved a new transdermal T system that is applied daily as a gel [19]. The T gel system causes less skin irritation and the dosage

may be adjusted to provide higher serum T levels than the patch systems. Therefore, the T gel system should be studied as a potential androgen for a male contraception regimen.

3.1.4 A non-reducible androgen: 7 α -methyl-19-nortestosterone

A synthetic androgen, 7 α -methyl-19-nortestosterone (MENT), might be an ideal androgen for a male hormonal contraceptive regimen. Although T is converted to dihydrotestosterone (DHT) by 5 α -reductase, MENT is resistant to enzymatic 5 α -reduction. Since DHT is the primary androgen involved in stimulating prostate growth, baldness and acne, MENT might provide the positive androgenic effects of T with fewer undesirable effects. A recent study of castrated macaque monkeys confirmed this hypothesis. MENT was 10 - 60 times more potent than T in gonadotropin suppression and anabolic effects but only twice as potent in stimulating prostate growth [20]. Thus, a small dosage of MENT could be used to suppress gonadotropins and spermatogenesis in men

while minimising androgen effects on prostate and skin.

The serum half-life MENT is very short because MENT does not bind to sex-hormone binding globulin and the elimination half-life of MENT after im. injection is only 4 h [21,22]. However, a study of a MENT acetate implant system showed that 2 or 4 subdermal implants suppressed serum gonadotropins $\geq 95\%$ for at least 1 month in healthy, young men [23]. No long-term studies of the effects of MENT on bone, muscle or sexual function have yet been published. In summary, long-acting formulations of MENT show great promise for an effective androgen-based male contraceptive that might confer additional health benefits such as prevention of baldness and prostatic hypertrophy.

3.1.5 Non-steroidal androgen receptor modulators

Recently, there have been several studies of new non-steroidal compounds that interact with the androgen receptor as agonists or antagonists [24-26]. These compounds have tissue-specific effects that result in selective androgen agonism or antagonism and many of them can be taken orally. These compounds have only been tested in animals but they represent exciting possibilities for oral male contraceptives as drugs that could be used to selectively suppress circulating gonadotropin levels and spermatogenesis without other androgen effects.

3.1.5.1 GnRH analogues

Experience from clinical trials has shown that exogenous T alone will not result in azoospermia in all men. Therefore, investigators have studied hormonal combinations that synergistically suppress gonadotropins and spermatogenesis such as T plus a GnRH analogue or progestin. GnRH agonists cause downregulation of GnRH receptors in the pituitary and are used clinically in men with advanced, metastatic prostate cancer to decrease in gonadotropin secretion with resultant suppression of steroidogenesis. However, studies of GnRH agonists for male contraception have been disappointing because the GnRH agonists that were studied did not uniformly suppress spermatogenesis, probably because of incomplete suppression of circulating gonadotropins.

GnRH antagonists such as Nal-Glu or Acyline induce more profound suppression of circulating gonadotropin levels and spermatogenesis than GnRH agonists and appear to be more promising for male contraception. Two studies have shown that T and Nal-Glu, a GnRH antagonist, is more effective than T enanthate alone in inducing azoospermia [27,28]. However, a third study demonstrated no clinically significant differences between combination therapy and T enanthate alone [29]. Another recent study showed that a short course of Nal-Glu and lower dosage T enanthate could induce azoospermia or oligozoospermia and that continuing T enanthate alone could maintain azoospermia or azoospermia in nearly all of the healthy, young subjects [30]. The maintenance dosage of T enanthate in this study was only 100 mg weekly, half the dosage of the WHO efficacy trials.

Although GnRH antagonists might be useful as part of a

male hormonal contraceptive, the older antagonists such as Nal-Glu have short half-lives and require frequent sc. injections. The older GnRH antagonists often cause local histamine release at the site of injection and may cause occasional sc. nodules that take weeks to resolve. Newer GnRH antagonists with longer half-lives have been developed. A single sc. injection of Acyline, for example, suppresses gonadotropins and sex steroid hormone levels to the lower limit of detectability for up to 1 - 2 weeks [31]. There were almost no local side effects beyond an occasional wheal that resolved within 90 minutes of the injection. In addition, an oral non-peptide GnRH antagonist has recently been described, but it has not been tested in humans yet [32].

3.3 Progestins

3.3.1 Short-acting preparations: levonorgestrel, desogestrel and cyproterone

Exogenous progestins suppress gonadotropin secretion and also might interfere directly with spermatogenesis [33] (Figure 1 and 3). In the past 10 years, randomised controlled trials have demonstrated that the combination of T and a progestin is more effective in suppressing gonadotropins and spermatogenesis than T alone [34-36]. In a randomised controlled trial, T enanthate (100 mg im. weekly) plus oral levonorgestrel (LNG) (500 μg daily) was superior to T enanthate alone in rapidly suppressing gonadotropins and achieving azoospermia (67 vs. 33%) [34]. However, this regimen was associated with weight gain and significant suppression of HDL cholesterol. A recently published follow-up study by the same group showed that T enanthate (100 mg im. weekly) plus lower dosages of oral LNG (125 or 250 μg daily) were equally effective in suppressing spermatogenesis but caused less weight gain and HDL-cholesterol suppression [37].

A study of transdermal T (5 mg daily by a non-scrotal patch) plus oral LNG (250 μg daily) yielded disappointing results [18]. Only 2/11 men became azoospermic and 5/11 suppressed to sperm concentrations < 3 million/ml. It is likely that the non-scrotal patch used in this study provided inadequate amounts of T to suppress gonadotropins and spermatogenesis. On the other hand, in another recent study im. T undecanoate (1000 mg every 6 weeks) plus oral LNG (250 μg daily) suppressed all 14 to oligozoospermia; 50% became azoospermic and the remainder suppressed to < 3 million/ml [8].

Recently, investigators have tested the combination of T plus desogestrel (DSG), a progestin that is thought to be less androgenic in women than LNG. The initial studies of T plus DSG were done at two different centres with slightly different protocols. The first study demonstrated that 50 or 100 mg of im. T enanthate weekly plus 300 μg of oral DSG daily or 100 mg of T enanthate plus 150 μg of DSG were all highly effective at suppressing gonadotropin levels and spermatogenesis [38]. Overall, 18/23 men became azoospermic and all but 1 suppressed to < 3 million/ml. In the second study, 100 mg of im. T enanthate weekly plus 150 or 300 μg of oral DSG daily induced azoospermia in 7/7 and 7/8 men, respectively [39].

Unfortunately, in both studies, T plus DSG resulted in significant HDL suppression that approximated or exceeded the decrease seen in T plus LNG studies.

The combination of T and cyproterone acetate (CPA) yielded particularly promising results in a small trial [36]. Groups of normal, young men were randomised to im. T enanthate (100 mg weekly) plus oral CPA (50 or 100 mg daily) or T enanthate alone. All men receiving CPA became azoospermic but only 3/5 in the T alone group became azoospermic. There was no change in serum HDL cholesterol levels, liver function tests or sexual function in the groups receiving CPA. There was a slight decrease in weight and haemoglobin that depended on the dosage of CPA. The optimal contraceptive dosage of CPA appears to be ~25 - 50 mg daily [40]. The superior effect achieved by the addition of CPA might depend on a dual inhibitory effect on spermatogenesis. In addition to suppressing gonadotropin secretion, it has been suggested that CPA directly inhibits spermatogenesis by competitively binding the androgen receptor in the testes [33].

In a different trial, the same group tested the first completely oral male hormonal contraceptive [16]. Unfortunately, the combination of oral T undecanoate (80 mg b.i.d.) plus CPA (12.5 mg daily) did not cause enough spermatogenic suppression for effective contraception. However, it is still possible that adjustments in this regimen (such as a sustained released form of T undecanoate, or some other safe oral androgenic compound plus CPA) could be effective.

3.3.2 Depot T - depot progestin combinations

Two male contraceptive studies have been conducted with long-acting T-progestin combinations. In one study, a single implantation of long-acting sc. T pellets (800 mg) plus depot medroxyprogesterone (DMPA; 300 mg) was significantly more effective than T pellets alone in achieving azoospermia (90 vs. 40%) [35]. Another long-acting progestin that has been recently studied is norethisterone enanthate (NETE) [41]. Intramuscular T undecanoate (1000 mg) plus im. NETE (200 mg) every 6 weeks induced azoospermia in 13/14 normal young volunteers [42]. In the studies of T +, DMPA or NETE, the most significant side effects were weight gain and HDL cholesterol suppression. These studies of T + DMPA or NETE confirm the likelihood that a long-acting androgen-progestin could be an effective male contraceptive.

4. Other factors

4.1 Acceptability

Clinical trials indicate that investigators are on the brink of designing effective, safe and reversible male hormonal contraceptives. Sceptics, however, have questioned whether men would use a hormonal contraceptive. A recent survey of almost 2000 men in Scotland, South Africa and China (Hong Kong and Shanghai) indicated that the majority of men would welcome the possibility of a male contraceptive pill [43]. Furthermore, at all sites except in Scotland, men thought that

women bore too much of the responsibility for contraception. Thus, men from countries in Europe, Africa and Asia have indicated a willingness to use a male hormonal contraceptive.

4.2 Delay in contraceptive effect

All male hormonal regimens take at least 8 - 10 weeks to suppress sperm concentrations to levels associated with effective contraception. Although any delay in contraceptive effectiveness is undesirable, this delay is not significantly different than the time required to achieve full contraceptive effectiveness after vasectomy (median time to azoospermia is ~10 weeks) [44]. Female hormonal contraceptives are also associated with delayed contraceptive efficacy. For example, women are often advised to use other contraceptive methods for at least one month after starting an oral contraceptive. In fact, only barrier methods (e.g., such as condoms and diaphragms) are immediately effective.

4.3 Non-uniform spermatogenic suppression

Although clinical trials indicate that hormonal regimens can suppress sperm concentrations to levels associated with effective contraception in virtually all men, the goal of azoospermia (or at least severe oligospermia) in all men who use the contraceptive has not yet been achieved. It has been hypothesised that men who fail to achieve azoospermia or severe oligospermia might have persistent production of intratesticular DHT that maintains spermatogenesis [45]. However, two recent studies that included a 5 α -reductase inhibitor to block conversion of T to DHT did not show any additional spermatogenic suppression [14,46]. However, it is possible that the 5 α -reductase inhibitor that was used did not adequately suppress intratesticular DHT levels [14]. Another explanation for non-uniform azoospermia is that FSH levels have not been adequately suppressed in regimens tested so far [47].

Asian men who live in Asia are significantly more likely to suppress to azoospermia during treatment with hormonal contraceptives [1,2,48]. Although this observation might not apply to Chinese men living away from China, it is likely that male hormonal contraceptives would be particularly effective in a region that accounts for at least one-fifth of the world population.

4.4 Potential markets for a male hormonal contraceptive

The most likely men to use a male hormonal contraceptive would be men who are in a monogamous relationship where reversible contraception is desired, men in densely populated countries where public policy has dictated strict family planning and men whose female sexual partners are intolerant of female contraceptive methods. However, only condoms prevent sexually transmitted diseases. Male (and female) hormonal contraceptive methods do not obviate the need for condoms for people with multiple sex partners.

5. Conclusions

We need effective male contraceptives that are safe and fully reversible. High-dose T alone has been shown to be very effective. The combination of lower dosage T plus a second agent that synergistically suppresses gonadotropin levels leads to greater spermatogenic suppression and permits a lower dosage of T than T alone regimens. There are a number of combination hormonal regimens that suppress sperm concentrations in virtually all men to levels associated with excellent contraceptive effectiveness. These regimens have been shown to be safe and reversible in short-term studies (6 - 18 months). The primary side effects of these regimens have been modest weight gain and suppression of HDL cholesterol. Large-scale studies of the contraceptive effectiveness of male hormonal contraceptives should be done.

6. Expert opinion

Male hormonal contraception is in the fledgling developmental stage that high oestrogen-progestin oral contraceptive pills were when they were introduced for use in the 1960s. We need an oral contraceptive pill for men because they prefer a pill to an injectable option [43]. The best hopes for an oral male contraceptive lie in the combination of a sustained release T undecanoate or non-steroidal androgen receptor

modulator plus a progestin. Likely candidates for a progestin in an oral contraceptive include CPA, DSG and LNG. CPA has the greatest appeal because it has few androgenic side effects such as weight gain and suppression of serum HDL cholesterol.

Although many men would prefer to use an oral contraceptive, experience in contraception for women suggests that compliance is higher with an im. depot injection and that such a formulation is preferable for many men and women. The combination of depot preparation of an androgen plus a progestin that could be injected every 1 - 3 months is close to realisation. A long-acting GnRH antagonist such as acyline might be useful as a third agent in a depot preparation.

T undecanoate or T pellets plus LNG, DSG or NETE implants are the most likely current prospects for a depot regimen. However, a MENT ester would be an ideal androgen for depot formulation to use because it might have relatively little androgenic effect on the skin and prostate. In fact, MENT or other selective androgens might confer salubrious effects beyond contraception by reducing the trophic effects of endogenous sex steroid hormones on the prostate.

We can hope that a long-acting depot male hormonal contraceptive is available for public use in this decade and that improved regimens including oral formulations and formulations with selective androgen effects follow shortly thereafter.

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Hair Loss Remedies—Separating Fact From Fiction

Ilian Bandaranayake, BA; Paradi Mirmirani, MD

GOAL

To understand the validity of claims for hair regrowth products

OBJECTIVES

Upon completion of this activity, dermatologists and general practitioners should be able to:

1. Explain the efficacy of various hair regrowth products.
2. Describe the side effects of various hair regrowth products.
3. Advise patients of the hair regrowth products most appropriate for them.

CME Test on page 123.

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This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of Albert Einstein College of Medicine and Quadrant HealthCom, Inc. Albert Einstein College of Medicine

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This activity has been planned and produced in accordance with ACCME Essentials.

Ms. Bandaranayake and Dr. Mirmirani report no conflict of interest. The authors report discussion of off-label use for tretinoin, spironolactone, Yasmin, dutasteride, and laser light therapy.

Hair loss is a common complaint in the outpatient setting. Frequently, patients conduct their own research on hair loss diagnosis and treatment and are faced with a number of manufacturers' claims that their products will benefit hair

loss. This paper explores the truth behind those claims of hair regrowth. We intend for this information to serve as a "consumer report" for healthcare providers and patients and to help separate some of the valid claims for hair regrowth from those that are purely fiction.

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Androgenetic alopecia (AGA) is a common patient complaint, affecting approximately half of all men and women by the age of 50 years.¹ Hair loss often can have significant negative effects

on self-esteem and body image.² Clinicians may be bombarded by questions from patients who have information about hair loss remedies from the Internet or testimonials from friends. However, it may be difficult or time-consuming for doctors to learn about the dozens of products claiming to promote hair growth and to then appropriately counsel their patients. In this article, we have interposed information obtained from the Internet with that obtained from peer-reviewed journals, when available, to support or refute claims made by the manufacturers or marketers of various products. We intend for this information to serve as a "consumer report" for healthcare providers and patients and help to separate some of the valid claims for hair regrowth from those that are purely fiction.

FDA-Approved Hair Loss Remedies

Minoxidil—In 1988, the US Food and Drug Administration (FDA) approved minoxidil 2% topical solution for use in treating AGA in men. A 2% solution marketed toward women became available in 1991, and a 5% solution for use in men became available over-the-counter in 1997.³ Since that time, generic formulations of minoxidil topical solution also have become available. Minoxidil is a vasodilator and a potassium channel opener,³ but its mechanism of action in promoting hair regrowth is unknown and appears to be independent of its vasodilation properties.³⁻⁷ The most common adverse side effects of minoxidil topical solution include scalp irritation, which occurs in about 7% of patients using the 2% solution, and hypertrichosis, which is noted in women.³ Because there are both generic and brand-name formulations of minoxidil topical solution, the cost of this therapy varies depending on which solution patients choose. However, most consumers pay between \$10 and \$20 for a 1-month supply.⁸

Minoxidil 2% topical solution has been proven to be effective both in stimulating new hair growth and in helping to prevent continued hair loss in both sexes.³ A recent study comparing minoxidil 2% and 5% topical solution in men showed that the men using the extra-strength formulation had 45% more hair regrowth after 48 weeks and an earlier response to the drug.⁹ A study published in 1992 showed that a year's treatment with minoxidil 5% was effective in improving hair density in 9 women,¹⁰ though the company has not yet obtained FDA approval for the use of this concentration in women. Minoxidil topical solution has even been found to be effective

and safe in adolescents, which is an important finding because AGA can occur in older children or teenagers and can cause significant psychological distress.¹¹

Some Web sites are claiming that products used in combination with minoxidil can increase the drug's efficacy. For example, the Hair Loss Control Clinic Web site claims that sebum on the hair follicle at the level of the scalp prevents minoxidil's penetration into the hair follicle, causing it to be absorbed ineffectively.¹² The site also claims that the high alcohol content of over-the-counter minoxidil can cause hair damage. The clinic is promoting a product called HLCC Scalp Therapy Dexpanthenol 12% to be used prior to shampooing to dissolve sebum, theoretically allowing the minoxidil solution to better penetrate the hair follicle.¹² Another product advertised on this site is Carrier Enhancement Agent, which supposedly neutralizes the alcohol in minoxidil to prevent scalp irritation.¹² There are no peer-reviewed studies supporting these claims. Because it is unclear how minoxidil topical solution works, it also is unclear why such additives would increase its effectiveness.

Patients also may see Web sites promoting the use of retinoids with minoxidil to enhance minoxidil's effectiveness. To date, one nonblinded study tested tretinoin 0.025% combined with minoxidil 0.5% topical solution in 36 patients and showed that the tretinoin increased the percutaneous absorption of the minoxidil. The combination of the 2 drugs led to visible hair growth in 66% of the patients tested.¹³ Therefore, the application of both topical minoxidil solution and tretinoin may give some patients better results than application of topical minoxidil alone. Patients may want to try minoxidil by itself at first and then add the tretinoin only if they are not satisfied with their initial results.

Finasteride—In 1997, finasteride was approved by the FDA for treatment of male AGA at a dose of 1 mg/d.³ This medication is a competitive inhibitor of type-2 5 α -reductase, which inhibits testosterone's conversion to dihydrotestosterone (DHT).¹⁴⁻¹⁷ Finasteride is able to decrease serum DHT by about 70%.¹⁸ Due to the potential for teratogenic effects in male fetuses, finasteride is not FDA approved for use in women.³ The main side effects of finasteride therapy are sexual side effects such as decreased libido and erectile/ejaculatory dysfunction, which occurred in fewer than 2% of men in one trial. In one study, these negative side effects were reversed with cessation of the medication.¹⁹ Also, there have been no clinically

significant drug interactions noted between finasteride and other medications.²⁰ The cost of this drug averages between \$30 and \$60 per month.⁸

Multiple randomized double-blind clinical trials of finasteride versus placebo in men aged 18 to 41 years with both vertex and frontal hair thinning showed that patients who took finasteride 1 mg/d for one year had significantly increased scalp coverage and hair counts than patients taking placebo.^{19,21,22} With continuous treatment of finasteride daily for 2 years, approximately two thirds of men have improved hair regrowth, one third of men see no change, and approximately 1% of men actually have less hair than at baseline.³ This product has shown more efficacy in younger men than it has in men older than 60 years, most likely because of decreased scalp type-2 5 α -reductase activity in older men.³

Finasteride also has been tested for efficacy in women with AGA. A randomized double-blind study of 137 postmenopausal women with AGA who took finasteride 1 mg/d or placebo for one year showed no significant difference in hair count between the 2 groups. In fact, both groups of patients actually showed significant loss of hair during the study period.²³ Another randomized open-label study tested finasteride 5 mg/d versus no treatment in premenopausal women with hyperandrogenic alopecia and elevated serum androgens (levels >2 SD above the mean in ovulatory control patients). This study found that the women using finasteride at this elevated dose did not see any significant improvement as opposed to the women receiving no treatment.²⁴ Some clinicians have had more success with the use of finasteride in their female patients with AGA. In a letter published in the *British Journal of Dermatology*, 2 physicians describe successful treatment of AGA in a postmenopausal woman who was given finasteride 5 mg/wk. Success was measured via patient report of improved hair density and review of stereotactic photographs of the scalp.²⁵ Despite this anecdotal evidence of success, the larger body of evidence weighs against the use of finasteride in postmenopausal women with AGA.

Because there are only 2 FDA-approved treatments for AGA, patients may wonder which is more effective. In a recent letter published in the *Archives of Dermatology*, finasteride 1 mg/d was compared with topical minoxidil 2% in 99 men aged 18 to 45 years with mid frontal and/or vertex hair thinning.²⁶ The researchers found that both treatments worked equally well in stopping the progression of hair loss in patients; however,

patients given minoxidil had quicker initial improvement whereby patients given finasteride had slightly better results as treatment progressed.²⁶ Choice of treatment therefore may be more a factor of side-effect profile, expense, and preferred form of medication (oral vs topical).

Some patients may ask about combining finasteride with minoxidil. One case study described the improvement of one man's alopecia from Hamilton-Norwood class V to class III after using a combination therapy of finasteride 5 mg/d plus a topical solution of minoxidil 3% and tretinoin 0.01%.²⁷ The combined use of finasteride and minoxidil topical solution has been studied in the animal model of AGA; in stump-tail macaques, the combined use of finasteride with minoxidil had greater effects on hair loss than either treatment alone.²⁸ Because both treatments have different modes of action, it is plausible that combining them may yield better results.

Off-Label Uses for FDA-Approved Medications

Spironolactone—This medication is often prescribed for the treatment of hypertension because of its action as an aldosterone antagonist, but it also is able to inhibit the biosynthesis of androgens and to competitively inhibit androgen receptor protein binding.²⁹ The main side effects of this medication are menstrual irregularities, hyperkalemia, gynecomastia in men, and gastrointestinal distress.²⁹ Women using this medication must be warned about the potential for feminization of male fetuses if pregnancy occurs during the course of treatment.³⁰ The cost of this medication at a dose of 200 mg/d is approximately \$60 per month, though it typically is covered by insurance policies.⁸

Spironolactone has shown efficacy in treating women with hirsutism,³¹ and it also may have mild efficacy in treating AGA at a dose of 200 mg/d.³² One study examining the efficacy of spironolactone in women with AGA showed that the women taking the medication had less hair loss than control patients after one year, but the women taking spironolactone still did not have more hair after treatment than at the start of the study.³³ Another study that examined the use of spironolactone 200 mg/d in 2 men and 2 women with AGA showed that the patients had an increase in the number of hairs in anagen phase from 22% at baseline to 84.5% at the end of 6 months of treatment.³⁴ Because this medication only has weak evidence for its use as a treatment for hair loss, clinicians should consider this medication

only in addition to other, more proven, means of therapy.

Yasmin®—This is an oral contraceptive pill composed of ethinyl estradiol and drospirenone, an analogue of spironolactone. Each pill contains drospirenone 3 mg, which is equivalent to spironolactone 25 mg.³⁵ According to the manufacturer of Yasmin, this oral contraceptive antagonizes androgen receptors without affecting sex-hormone-binding globulin synthesis or affecting the binding of testosterone to sex-hormone-binding globulin. The manufacturer also claims that the drug inhibits ovarian androgen production.³⁶ The most common side effects are similar to side effects of other oral contraceptives and include breast tenderness, nausea, headache, emotional lability, dysmenorrhea, intermenstrual bleeding, and depression.³⁵ Some insurance plans will cover the cost of oral contraceptives, but for patients paying out of pocket, Yasmin costs approximately \$30 per month.⁸

Because spironolactone is sometimes prescribed for AGA, some clinicians recommend Yasmin to patients with alopecia who also are looking for effective contraceptive methods. However, to our knowledge, there are no known published studies showing that Yasmin prevents hair loss or promotes hair regrowth. Because spironolactone has shown only slight efficacy in treatment of women with AGA,³ it is unclear what the effect of Yasmin may be on hair loss. However, this may be a reasonable choice of contraceptive in a woman with AGA.

Dutasteride—This new 5 α -reductase inhibitor blocks both type-1 and type-2 isoenzymes.³⁷ By inhibiting both types of 5 α -reductase, dutasteride is able to achieve a greater than 90% suppression of DHT.³⁷ This medication was developed for the treatment of benign prostatic hyperplasia, with side effects similar to those of finasteride.³⁸ As with finasteride, women are advised not to take this product because of the potential risk of birth defects in male fetuses.³⁷ In November 2002, dutasteride was approved by the FDA for use in patients with benign prostatic hyperplasia.³⁹ This medication costs approximately \$75 for a 1-month supply.⁸

Although dutasteride is not yet FDA approved for alopecia, the manufacturers have completed phase 2 clinical trials of the medication for the treatment of hair loss and are hopeful it will be approved by the FDA in 2006.⁴⁰ There are no studies published regarding this medication's effect on AGA, but preliminary results from the manufacturer showed that dutasteride reduced scalp DHT in men to a greater extent than finasteride.⁴¹

Herbal/Dietary Remedies

Saw Palmetto—Saw palmetto, or *Serenoa repens*, is an herbal remedy that is processed from fruit of the American dwarf pine tree.⁴² It often is used to treat benign prostatic hypertrophy because of its ability to inhibit 5 α -reductase levels by 32% without affecting testosterone levels in men.⁴³ Extracts of saw palmetto also have been shown to have a partial antagonistic effect on testosterone receptors.⁴⁴ It is most likely that these 2 actions led to saw palmetto being used as a hair loss remedy. Saw palmetto is believed to be a safe herbal supplement, with a primary side effect of mild gastrointestinal distress.⁴² Also, clinical trials conducted in human patients showed that consumption of saw palmetto supplements did not result in any clinically significant alterations in laboratory parameters.⁴⁵ Saw palmetto has no known drug interactions.⁴² The cost of this supplement varies by manufacturer, but consumers should be able to find saw palmetto supplements for as little as \$3 for a month's supply.⁸

One double-blind placebo-controlled study examined saw palmetto's effect on AGA.⁴⁶ In this study, researchers studied the efficacy of a softgel containing β -sitosterol 50 mg and saw palmetto 200 mg extract (components of the HairGenesis™ Softgels discussed later) versus placebo in treating AGA. They found that 60% of patients taking the active softgel rated their hair growth as improved from baseline as opposed to only 10% of the patients taking placebo. However, this study had a limited patient population and also concurrently tested β -sitosterol, so any improvement cannot be attributed to saw palmetto alone.

Biotin—This is a water-soluble B complex vitamin that is used in the body as a cofactor for biochemical carboxylations. Patients that are deficient in this vitamin often have alopecia, brittle nails, and a scaly erythematous dermatitis.^{47,48} Biotin is water-soluble, and there are no known side effects of supplementation and no documented cases of biotin overdose.⁴⁹ As with other supplements, cost of treatment will depend on the manufacturer, but consumers should be able to find biotin for as little as \$2 for a month's supply.⁸

Dietary supplementation with biotin has been shown to improve the clinical condition of brittle nails,⁴⁸ but no studies have been conducted looking at biotin's effect on AGA. Although it is true that biotin deficiency can lead to alopecia, such a deficiency has not been demonstrated in healthy humans eating a mixed diet.⁴⁷ The only 2 situations in which human biotin deficiency has

been demonstrated are in patients with extended consumption of raw egg whites⁵⁰⁻⁵² and in patients with malabsorption syndromes receiving parenteral nutrition without biotin supplementation.^{50,53} Supplementing the diet with biotin is unlikely to harm a patient, but there is no data to suggest any improvement in hair regrowth.

Other Hair Regrowth Products

Avacor™—Sold through the Internet and directly from the manufacturer, Avacor is a hair regrowth product marketed toward both sexes for treatment of AGA.⁵⁴ The product line consists of a scalp detoxifying shampoo, an herbal supplement, and a topical solution. The purpose of the shampoo as stated by the manufacturer is to deep clean the scalp to improve the absorbency of the topical treatment. The herbal supplements are to be taken twice daily to “maintain a healthy hair follicle” and consist of bilberry, ginkgo biloba, saw palmetto, and horsetail. The topical solution, which is marketed to men only, claims to dilate blood vessels in the scalp, allowing increased nutrient and oxygen delivery to the scalp. The Web site claims that these products must be used together and that they have no known side effects.⁵⁴ The cost of this product is \$239.95 for a 3-month supply.⁵⁵

The official Avacor Web site has a summary of a clinical study performed by The New York Hair Clinic and the Hair and Skin Treatment Center in which 200 men aged 18 to 65 years used the 3-part system for 24 weeks.⁵⁵ The Web site claims that 91% of the men had a decrease in hair loss and an increase in strength and thickness of preexisting hair within 3 months.⁵⁵ However, this study does not appear to be published in any journal and consumers can only receive a copy of the study if they purchase the product.

A *Wellness Letter* highlighting dietary supplements, published by the University of California at Berkeley, showed that Avacor contains minoxidil in its topical solution despite its claims to be made from only natural ingredients.⁵⁶ In April 2003, the FDA sent the makers of Avacor a letter informing them that their products are considered drugs under section 201(g) of the Federal Food, Drug, and Cosmetic Act and should have had an approved New Drug Application prior to being marketed in the United States. The FDA also points out that the 3 individual components are mislabeled because the active ingredients are not listed on their labels.⁵⁷

Given the lack of peer-reviewed evidence of hair growth and with all of the controversy surrounding

this product, it would not be wise for clinicians to recommend this therapy to any patient.

HairGenesis™—This product line consists of 4 items: Revitalizing Oral Softgel™ supplements (β -sitosterol 50 mg and saw palmetto 200 mg extract) that claim to strengthen and protect hair; Topical Activator Serum that consists of various 5 α -reductase inhibitors; Hair Revitalizing Formulation, a shampoo that has similar components to the Topical Activator Serum; and Hair and Scalp Conditioner that also is meant to strengthen and protect hair. Although the company states that the products may be used individually, it recommends using them all synergistically, at a cost of \$200 for a 3-month supply.⁵⁸ The efficacy of the oral softgel containing saw palmetto is discussed above; there are no known research studies published about the other 3 components of the HairGenesis system.

Nioxin®—This product line is sold only through hair salons and does not aim to regrow hair; rather, it claims to “create an optimum scalp environment” for regrowth and maintenance of the current hair count. The manufacturer claims to accomplish this by clearing the scalp of excess sebum that may contain high levels of DHT. Ingredients include various vitamin-B coenzymes, biotin, saw palmetto, aloe, ginseng, and amino acids. The manufacturer claims that the Nioxin system has no known side effects.⁵⁹ As this product is sold only in salons, the cost for a month's supply will vary depending on the place of purchase. One salon that we contacted offered a one-month starter kit for \$30. The manufacturer does not disclose its clinical studies but claims that its studies are conducted by “world-renowned” researchers who are experts in hair thinning.⁵⁹ However, there is no known published scientific evidence that any of the ingredients in Nioxin are effective in treating hair loss or maintaining hair count, or that excess sebum leads to hair thinning.

Laser Light Therapy—Low-intensity laser light therapy has been shown to be effective in promoting wound healing⁶⁰ and in improving circulation.^{61,62} For these reasons, some hair loss treatment centers are offering the use of lasers for treating alopecia in both men and women. To date, there are no known studies looking at the efficacy of these lasers for treating hair loss. The use of low-intensity laser light for treating alopecia is FDA approved for safety only, not for efficacy.²⁹ This therapy is expensive, costing as much as \$3500 for the recommended 6 months of treatment.⁶³

Although various Web sites claim efficacy based on double-blind placebo-controlled studies of laser light treatment versus placebo laser treatment,^{64,65} such studies are not available for viewing anywhere on the Web sites. To the best of our knowledge, there are no peer-reviewed articles supporting efficacy of this type of treatment for AGA. Until reliable evidence of the effectiveness of laser light therapy for alopecia is published, this treatment remains experimental, at best.

Conclusion

Any consumer looking on the Internet for a treatment for hair loss is exposed to a multitude of remedies. However, only the FDA-approved treatments for AGA, finasteride and minoxidil, have any well-studied factual evidence of efficacy. Smaller studies have shown possible benefit of combining topical tretinoin with minoxidil, as well as combining finasteride and minoxidil. Spironolactone in high doses (100–200 mg), dutasteride, and saw palmetto, also may provide benefit; however, larger studies are needed to consider these agents as first-line treatments for AGA. In addition to efficacy, clinicians need to consider patient preferences, safety profile, and cost when counseling patients about treatment options for AGA.

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Anabolic-Androgenic Steroid Therapy in the Treatment of Chronic Diseases

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The purpose of this study was to review the preclinical and clinical literature relevant to the efficacy and safety of anabolic androgen steroid therapy for palliative treatment of severe weight loss associated with chronic diseases. Data sources were published literature identified from the Medline database from January 1966 to December 2000, bibliographic references, and textbooks. Reports from preclinical and clinical trials were selected. Study designs and results were extracted from trial reports. Statistical evaluation or meta-analysis of combined results was not attempted.

Androgenic anabolic steroids (AAS) are widely prescribed for the treatment of male hypogonadism; however, they may play a significant role in the treatment of other conditions as well, such as cachexia associated with human immunodeficiency virus, cancer, burns, renal and hepatic failure, and anemia associated with leukemia or kidney failure. A review of the anabolic effects of androgens and their efficacy in the treatment of these conditions is provided. In addition, the

numerous and sometimes serious side effects that have been known to occur with androgen use are reviewed.

Although the threat of various side effects is present, AAS therapy appears to have a favorable anabolic effect on patients with chronic diseases and muscle catabolism. We recommend that AAS can be used for the treatment of patients with acquired immunodeficiency syndrome wasting and in severely catabolic patients with severe burns. Preliminary data in renal failure-associated wasting are also positive. Advantages and disadvantages should be weighed carefully when comparing AAS therapy to other weight-gaining measures. Although a conservative approach to the use of AAS in patients with chronic diseases is still recommended, the utility of AAS therapy in the attenuation of severe weight loss associated with disease states such as cancer, postoperative recovery, and wasting due to pulmonary and hepatic disease should be more thoroughly investigated. (*J Clin Endocrinol Metab* 86: 5108-5117, 2001)

ANDROGENIC STEROIDS such as T and its derivatives have a wide range of uses in clinical medicine and were initially recognized for their anabolic effects. In 1889, French physiologist Charles Edouard Brown-Sequard announced that an extract of dog and guinea pig testicles given iv results in an increase in physical strength, improvement in intellectual energy, relief of constipation, and lengthening of the arc of his urine. In the late 1930s the anabolic agent responsible for these effects, the androgens, were isolated. In the 1940s, scientists confirmed Brown-Sequard's claim that androgens, particularly T, could facilitate muscle growth. With the publication in 1945 of Paul de Kruif's widely read book, *The Male Hormone*, T use among athletes became more common. Although initially used by body builders, the positive results encouraged AAS use in other strength-intensive sports, including football, track and field, hockey, swimming, soccer, cycling, volleyball, and wrestling.

Anabolic-androgenic steroids (AAS) have also been used in clinical practice since the 1940s in the treatment of chronic debilitating illnesses, trauma, burns, surgery, and radiation therapy (1-4). The effects on hematological parameters were recognized as early as 1942, and before bone marrow trans-

plantation and the use of synthetic erythropoietin became common, AAS were often used to treat various types of anemias (5). Noréthandrolone and methandrostenolone [Dianabol (discontinued in 1993); CIBA, New Jersey] also became available on the market during the 1950s. The psychoactive effects of AAS broadened its use to treat depression and melancholia.

Recent studies demonstrating positive effects of AAS on body composition have prompted further research in their use in treating the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS)-associated wasting syndrome. Since 1995, the use of AAS is estimated to have increased 400%, mostly attributable to treatment of AIDS-associated wasting. However, cachexia is prevalent in a wide spectrum of chronic diseases, including chronic renal failure, hepatic cirrhosis, cancer, and pulmonary disease. Although increased caloric intake and an exercise regimen are of paramount importance in the maintenance of body weight, treatment with anabolic agents may enhance the effects of these measures.

AAS therapy does have several clinical uses other than androgen replacement. These compounds are used in the treatment of short stature (as in Turner's syndrome or constitutionally delayed growth and puberty), breast cancer (as an anti-estrogen), and the treatment of hereditary angioedema. These applications are not discussed in this review. Instead, we focus primarily on the anabolic properties of these agents in patients with debilitating conditions.

Abbreviations: AAS, Androgenic anabolic steroids; AIDS, acquired immunodeficiency syndrome; COPD, chronic obstructive pulmonary disease; HDL, high density lipoprotein; HIV, human immunodeficiency virus; LBM, lean body mass.

AAS chemistry

Synthesis. T is a steroid hormone synthesized primarily in the Leydig cells of the testes in men; however, it is also present in women, in whom it is synthesized in the ovaries and adrenal glands. Its synthesis is stimulated by the action of LH, which in men, targets testicular Leydig cells resulting in an increase in cAMP production. This, in turn, enhances the activity of the enzymes needed for T synthesis and increases the availability of their primary substrate, cholesterol. This is followed by a cascade of enzymatic reactions that yields T as the final product.

Healthy adult men produce between 2.5–11 mg T/d, with plasma concentrations ranging between 300–1000 ng/dl (1). About 44% of the secreted T is bound to SHBG, whereas about 2% is in free form. The remaining T (54%) is known as bioavailable T, which is loosely bound to albumin and can dissociate within capillary beds (2). In target tissues such as the prostate, seminal vesicles, and pubic skin, T is irreversibly converted to DHT by 5 α -reductase. DHT has a relatively higher receptor binding affinity of 0.46 compared with 0.23 of T (3). Estimates of the relative potency of DHT to T have ranged from 2:1 to 10:1. Likewise, the dissociation constant for DHT is 0.25–0.50 nM, while the K_d of T is 0.4–1.0 nM, indicating that DHT is a much stronger androgen.

In women, T is secreted by the ovaries and adrenal glands. About 50% of the secreted T (0.25 mg/d) is synthesized extraglandularly, where androstenedione produced by the adrenals is converted to T. Plasma concentrations range from 15–65 ng/dl. The majority of T produced in women is converted to E2 in adipocytes by the enzyme aromatase.

Classification. Since T in its native form is rapidly absorbed and degraded regardless of the route used, the use of modified analogs has become a favored method of androgen administration. There are three main classes of androgen analogs. Class A is made up of those analogs produced via esterification of the 17 β -hydroxyl group with any of the

several carboxylic acid groups. Longer carbon chains in these groups yield androgen derivatives that are more soluble in lipid vehicles, such as those used for im injection. T, when injected as a solution in oil, is rapidly absorbed, metabolized, and excreted. T esters are less polar and are absorbed slowly when injected im in oil. Different esters have variable durations of action, and therefore the frequency of T administration depends on the type of ester being used. T propionate is given two or three times weekly, T cypionate and enanthate are effective when given at 2- to 4-wk intervals, and T buciclate can be administered at 12-wk intervals (6). Class B analogs are those that have been alkylated at the 17 α position, such as methyltestosterone. Class C analogs are those that are produced via modification of the A, B, or C rings, such as mesterolone. These analogs often exist in conjunction with those of class A as AC analogs (Table 1). As alkylated analogs and those with a modified ring structure are not metabolized by the liver as quickly as T and its 17 β -esterified derivatives, therefore, class B and C analogs are available for oral use (4).

Metabolism. T is inactivated primarily by the cytochrome P450 family of hepatic isoenzymes. Therapeutic preparations of T have been developed to circumvent this immediate metabolism. Class A derivatives have long alkyl side-chains, rendering them less polar than T and hence retarding their hepatic metabolism and increasing their half-life in the peripheral tissues. They are, however, eventually hydrolyzed and metabolized by the same pathway as endogenous T. The modification in the class B and C derivatives alters their metabolic pathway, yielding a longer half-life. They are variably excreted either unaltered or as metabolites and conjugates in the urine or feces (5).

Is there a pure anabolic or androgenic agent?

For decades researchers have known the anabolic potential of androgens. This made the use of androgens popular

TABLE 1. T analogs approved in the U.S.

Drug class	Generic name	Trade name	Route	Indications	Dosage
A	T propionate	Testex	im	T replacement	10–25 mg 2–3 \times /wk
	T enanthate	Delatestryl, Everone, Durathate	im	T replacement	50–400 mg every 2–4 wk
	T cypionate	Virilon im, Depotest, Andro-Cyp	im	T replacement	50–400 mg q 2–4 wk
	T patches	Androderm, Testoderm TTS	Top	T replacement	5 mg/day
	T Gel	Androgel	Top	T replacement	5 g/day
AC	Nandrolone decanoate	Deca-Durabolin	im	Renal insufficiency-associated anemia	50–200 mg/wk
	Nandrolone, phenpropionate	Durabolin	im	Renal insufficiency-associated anemia	50–200 mg/wk
B	Methyltestosterone	Testred, Android, Virilon	PO	T replacement; endometriosis	10–50 mg/d 800 mg/d initially
BC	Danazol	Danocrine	PO	HAE	400–600 mg/d initially
	Fluoxymesterone	Halotestin	PO	T replacement	5–20 mg/d
	Methandrostenolone	Methandienone	PO		
	Oxandrolone	Anavar, Oxandrin	PO	Wt loss	5–10 mg/d
	Oxymetholone	Anadrol	PO	Anemia	1–5 mg/kg/d
	Stanozolol	Winstrol	PO	HAE attack prevention	6 mg/d

HAE, Hereditary angioedema; PO, taken orally.

among athletes. However, it was soon noted that these agents along with being anabolic, also result in androgenic side effects such as acne and increased sebum production in men and hirsutism and even virilization in women. For years, scientists have labored to dissociate anabolic from androgenic effects with the hope of producing a purely anabolic agent that is free from any androgenic side effects. Unfortunately, to date no such compound exists.

Androgens are a group of biologically diverse compounds with a variety of effects (anabolic and androgenic) in different body tissues (7). The androgenic effects of AAS include induction of male phenotype starting from sexual differentiation *in utero*, growth of sexual organs (genitals and prostate), development of secondary sexual characteristics, maintenance of sexual function, and fertility. The anabolic effects of AAS include nitrogen retention and increases in muscle mass and strength.

Although androgens mediate a broad range of developmental and homeostatic function, all of the androgens induce their response via a single AR despite this diversity. The receptor is a 120-kDa cytosolic protein encoded on the X chromosome, and to date only one AR cDNA has been identified (8). As there is only one AR, how do AAS mediate these diverse actions? Attempts in the past have failed to isolate a pure anabolic or a pure androgenic receptor (9). One explanation in the case of T is that it is a prohormone, and many of its actions in different tissues are mediated by its metabolites. T is converted by 5 α -reductase to DHT (the main androgen in the prostate) and by aromatase to E2. It is known that skeletal muscle is almost devoid of 5 α -reductase activity, and therefore T is the major hormone in the skeletal muscle promoting anabolism (9). Furthermore, the relative binding affinity of DHT to AR in muscle is much lower than that to AR in the prostate. On the other hand, prostate tissue is rich in 5 α -reductase activity, and almost whatever T enters the prostate is converted to DHT, which maintains its growth along with that of seminal vesicles and vas deferens, hence exerting its androgenic action. These analogs interact with the AR directly. These data show that the conversion of AAS in various tissues into different metabolites and the relative binding affinity of these metabolites to AR in these tissues are responsible for its diverse actions. However, in a recent animal study, Hsiao *et al.* (10) found two different kinds of androgen response elements that could respond differentially to T and DHT. Therefore, it is possible that a selective androgen response element sequence may play a role in differential T vs. DHT AR *trans*-activation.

Mechanism of anabolic action of AAS

Many studies have shown that administration of androgens to hypogonadal young and elderly men results in an increase in lean body mass (LBM) (11, 12). However, interestingly, supraphysiological doses of T result in an increase in muscle mass and strength even in eugonadal men (13). The positive response observed in these men (even though the majority of ARs are likely to be saturated) suggests that androgens also mediate anabolic effects indirectly, *i.e.* not via AR. Therefore, one can divide the anabolic actions of AAS into direct and indirect mechanisms.

Direct mechanism

Administration of T to hypogonadal men results in an increase in both contractile and noncontractile skeletal muscle proteins. Increased incorporation of leucine into the skeletal muscle was observed in six hypogonadal men after 6 months of treatment with T cypionate (14). There was a 56% increase in the fractional synthetic rate of mixed muscle proteins from the baseline, including a 46% increase in the synthesis of myosin heavy chain, the main contractile protein (14). All men had an increase in muscle mass from the baseline. Similarly, short-term administration of oxandrolone (a synthetic analog of T) to normal young men resulted in a 44% increase in the fractional synthesis of muscle proteins (15). Furthermore, oxandrolone administration significantly increased mRNA levels of skeletal muscle AR. Similarly, a single injection of 200 mg T enanthate results in increased skeletal muscle protein synthesis and efficient utilization of amino acids (16). In summary, androgens increase muscle mass and strength by increasing efficient utilization of amino acids and, at least in case of oxandrolone, by increasing AR expression in skeletal muscle.

Indirect mechanism

Antiglucocorticoid action. Indirect evidence exists that the anabolic effects of androgens on skeletal muscle may be mediated by the antiglucocorticoid action of androgens. *In vitro* experiments have shown that T has a high affinity for GR (17). The same group has also shown that T acts as an antagonist to endogenous circulating glucocorticoids (18). These observations are appealing because there is a great degree of homology between AR and GR (19). These observations are further supported by the fact that antagonism of glucocorticoids prevents muscle atrophy in men who have undergone orchidectomy (20). Furthermore, administration of large doses of AAS to these men result in an increase in urinary free cortisol (20). Men with androgen insensitivity syndrome also show nitrogen retention when given large doses of AAS despite having nonfunctional ARs (21). Similarly, T administration to patients with severe burns (a state of hypercortisolism and hypogonadism) shows a significant decrease in protein breakdown (22). Although the majority of the reports suggest GR antagonism as the main mode of androgen action, some have proposed that AAS interfere with glucocorticoid action at the gene level by interfering with hormone response elements (23).

Interaction with IGF-I system. Intravenous infusion of IGF-I results in stimulation of skeletal muscle protein synthesis (24). It has been shown that androgens are necessary for the local production of IGF-I within the skeletal muscle regardless of the systemic IGF-I levels and rate of GH production. This is supported by the observation that induction of hypogonadism in normal young men results in a reduction in IGF-I mRNA levels in skeletal muscle (25). Indeed, when hypogonadal elderly men are treated with T, there is an increase in IGF-I mRNA levels in muscle biopsy specimens (26). These reports show that T-IGF-I interaction is also important for the anabolic process.

Regulation of myostatin gene. The effects of AAS at the genetic level are currently poorly understood. Recently, the human myostatin gene was cloned. This gene is located on chromosome 2 and is a negative regulator of muscle growth. Inactivating mutations of this gene in mice and cattle are associated with double muscling in these animals (27). The myostatin protein is secreted into the serum and can be measured in the circulation. In a recent study, myostatin levels were elevated in the serum and skeletal muscle biopsy specimens of patients with AIDS associated sarcopenia compared with those in AIDS patients without any weight loss and normal controls (28). Furthermore, high levels of circulating myostatin have produced muscle atrophy in the rat (29). Preliminary research suggests that the myostatin protein may play a role in age-associated sarcopenia (30). This is further supported by the fact that low gravity-induced muscle wasting accompanies an increase in myostatin mRNA (31). As androgen levels decline with aging, it is possible that myostatin levels may rise as a result of andropause. Therefore, it is possible that androgens may exert their anabolic effects by either directly or indirectly suppressing the expression of myostatin. However, the role of myostatin in humans is not extensively defined, and these hypotheses should be tested by well designed clinical research.

Therapies currently in use for treatment of cachexia

Therapies that are currently available include AAS, megestrol acetate (Megace; Bristol-Myers Squibb, Princeton, NJ), GH, high calorie supplements, parenteral nutrition, and exercise. Therapy with Megace typically results in an increase in fat mass. GH use is associated with high cost and some untoward side effects. For this reason, other methods of treatment are in demand. Table 2 provides a summary of the conditions in which AAS therapy has been tested. These conditions are described below.

AIDS. Cachexia/anorexia resulting from an imbalance between nutritional intake and resting energy expenditure is a common problem in HIV patients. Weight loss of greater than 10% baseline body weight (HIV-associated wasting) is a strong predictor of mortality in HIV-infected men (32). The use of T and its analogs in the treatment of chronic disease-associated catabolism is best studied in HIV/AIDS patients,

because a significant proportion of this population has hypogonadism (33). Furthermore, the degree of weight loss in male HIV patients correlates with reductions in circulating T levels (34). This shows that androgen depletion may play a role in HIV-associated wasting.

Androgen replacement therapy for the treatment of HIV-associated wasting has met with varying degrees of success depending on the preparation, route of administration, and dosage used (35). Many studies using im T preparations to treat HIV-associated wasting have been performed to examine its effects on body composition. In an uncontrolled, open label study of T cypionate (400 mg every 2 wk), an average weight gain of 2.3 kg over a period of 12 wk was observed (36). Grinspoon *et al.* (37) have also shown an increase in LBM and muscle mass using 300 mg T cypionate every 3 wk. Bhasin *et al.* (38) recently completed a randomized, double blind, placebo-controlled 16-wk trial of T enanthate (100 mg/wk) and exercise (alone and in combination) compared with placebo in HIV-infected men with hypogonadism (total T, <350 ng/dl) and 5% weight loss. T-treated patients experienced a total weight gain of 2.6 kg and an increase in LBM of 2.3 kg. There was also a significant increase in muscle strength. The patients in the exercise-only group also showed an increase in total weight and LBM, whereas the placebo group lost weight. Interestingly, T and exercise in combination did not result in greater gains than either intervention alone. However, one trial with T enanthate did not produce any significant weight gain (39).

Transdermal T patches have also been used in HIV patients. The transscrotal T patch, Testoderm (Alza Corp., Mountain View, CA), at a dose of 5 mg daily did not result in weight gain or increase in LBM in HIV patients (40). On the other hand, Androderm (TheraTech, Inc., Salt Lake City, UT), a nonscrotal T patch, has been shown to increase LBM when applied at the same dose of 5 mg/d (41). It is important to remember that the difference in efficacy between different products could relate to the level of T achieved in the serum. Preparations achieving lower T levels demonstrated less significant benefit. Recently, Miller *et al.* (42) for the first time showed that therapy with T patches in women with HIV results in a significant improvement in weight and overall

TABLE 2. Efficacy of AAS therapy in chronic diseases associated with catabolic states

Condition	Wt gain efficacy	Disease-specific efficacy	Safety comments
HIV	Yes	No	
Pulmonary	Yes	Conflicting ^a	
Liver failure	Yes	Yes ^b	Hepatic dysfunction associated with 17 α -alkylated analogs
Postoperative recovery	Yes	Yes	
Burns	Yes	Yes ^c	
Cancer	Not studied ^d	Yes ^e	
Renal failure	Yes	Yes ^f	Sodium retention may exacerbate edema

^a Studies examining AAS effects on maximal inspiratory pressure (Pmax) have yielded conflicting results.

^b Oxandrolone treatment in alcoholic hepatitis has yielded significant improvement in liver function.

^c AAS has beneficial effects in preliminary studies.

^d The efficacy of AAS therapy for weight gain in cancer patients has not yet been examined in a clinical trial.

^e AAS therapy has been shown to have positive effects on remission rates in leukemia patients.

^f In addition to increasing lean body mass in dialysis patients, AAS also improves erythropoietin synthesis.

quality of life compared with placebo. Furthermore, no adverse effects of T patches were seen in women.

Oral preparations of T are seldom used because of rapid metabolism and inactivation in the case of class A analogs, and liver toxicity in the case of class B and C analogs. However, oxandrolone, an orally active T derivative, may be suitable for treatment of HIV-associated wasting. Significant increases in weight and LBM in patients treated with oxandrolone have been demonstrated. In a 4-month randomized, placebo-controlled study of oxandrolone (15 mg/d) in 63 AIDS patients with more than 10% body weight loss, oxandrolone resulted in significant weight gain, increase in appetite, and improvement in physical activity (43). At wk 16, patients taking oxandrolone had an increase of 0.6 kg in mean body weight, whereas the placebo patients lost 1.1 kg. In another study eugonadal HIV patients with weight loss were given T therapy at a dose of 100 mg/wk and randomized to oxandrolone (20 mg/d) or placebo (44). The patients in the oxandrolone group experienced increase in nitrogen retention, LBM, and muscle strength. Similarly, the use of nandrolone decanoate in this patient population also resulted in a significant increase in weight and LBM (45, 46). Oxymetholone is another oral preparation that has been used to treat HIV wasting with positive effects on total body weight (47). At a dose of 50 mg three times a day, oxymetholone resulted in an increase of 8.2 kg over a 30-wk period, whereas the subjects on placebo lost 1.8 kg. There was also a significant improvement in the quality of life variables in subjects taking oxymetholone.

In summary, these studies suggest that T and its analogs, regardless of the route of administration, result in an increase in weight and LBM. However, further studies would be welcomed to determine the exact nature of the relationship between factors such as dosage, route, and preparation used and the resultant changes in body composition in HIV patients, including women.

Pulmonary disorders. As in HIV, weight loss in patients with chronic obstructive pulmonary disease (COPD) is associated with mortality (48). Recent studies indicate a potential use for AAS therapy in COPD-associated wasting. A regimen of exercise, 250 mg im T administration at the baseline visit, and then 12 mg/d oral stanozolol for 27 wk showed significant improvement in weight, body mass index, LBM, and muscle size compared with exercise alone in patients with COPD (49). However, there was no increase in maximum inspiratory pressure or measures of physical endurance. Schols *et al.* (50) studied 217 patients with COPD and randomized them to either nandrolone decanoate plus nutrition and exercise or nutrition and exercise alone for a period of 8 wk. There was a significant increase in fat-free mass and an improvement in maximum inspiratory pressure in the nandrolone group. Similarly, oxandrolone therapy (20 mg/d) in tetraplegic patients produced significant improvement in weight and respiratory parameters (51). Caution is recommended when treating COPD patients with androgens due to the risk of developing polycythemia. Further research in a large number of patients is needed before the use of AAS becomes routine in this patient population.

Liver disease. AAS also have a role in treating patients with hepatitis-related malnutrition. In a study of 271 patients with alcoholic hepatitis, oxandrolone along with a high calorie supplement was compared with placebo and a low calorie supplement. Significant improvement in liver function and overall survival was observed in the oxandrolone and high calorie supplement group (52). Similarly, oxandrolone therapy has been shown to result in a reduction in 6-month mortality in patients with alcoholic hepatitis (53). In a V.A. cooperative study of 273 patients with moderate protein calorie malnutrition secondary to alcoholic hepatitis, 80 mg/d oxandrolone along with an enteral food supplement resulted in improved 6-month survival, decrease in liver injury, and improvement in malnutrition compared with the placebo group (54). However, no significant improvement was observed in patients with severe malnutrition. Although this dose of oxandrolone was very high, especially in a population with established liver disease, no hepatotoxicity was reported in subjects taking oxandrolone. In summary, although the preliminary studies hold promise, the use of AAS in these patients is not considered a standard of care and may be potentially dangerous. Further studies are necessary to fully characterize the effects of AAS, especially 17 α -alkylated agents such as oxandrolone, in this patient population.

Wound healing and postoperative recovery. The anabolic effects of T may also have a place in the process of wound healing and surgical recovery. The 17 α -alkylated agent stanozolol has been shown *in vitro* to significantly enhance collagen synthesis when applied to human dermal fibroblasts (55). Animals with full thickness wounds when treated with oxandrolone show early closure and increased tensile strength of the wound (56). A positive effect of AAS on wound healing in patients with nonhealing wounds has also been demonstrated (57). In this study patients with weight loss and nonhealing wounds for more than 1 yr who had failed to respond to nutritional supplements showed significant weight gain while taking oxandrolone. As they restored their body weights, there was significant improvement in the rate of wound healing, as measured by wound diameter. Amory *et al.* (58) have recently shown that the positive effects of AAS on muscle strength lead to early mobilization and, hence, alleviates the postoperative debilitation associated with knee replacement surgery. In their placebo-controlled trial of T enanthate (600 mg weekly for 3 wk before surgery), there was a significantly shorter in-patient stay and a higher degree of functional independence in patients receiving T. Although the data on wound healing appear promising, the process of wound healing may be due to general recovery and early mobilization of these patients rather than to direct effects of AAS on the wounds itself. Therefore, until more research is available AAS should not be used on a routine basis to expedite the process of wound healing.

Burns. There is a significant decrease in T levels in patients with severe burn injuries (59). As these patients are catabolic, the anabolic effects of AAS may play an important role in weight gain in these patients. The efficacy of AAS was tested in a prospective randomized study of 13 burn patients, 7 of whom received oxandrolone (10 mg twice daily) along with

a high protein diet, whereas the remaining 6 were treated with diet alone (60). There was no difference in daily caloric intake between the two groups. Patients taking oxandrolone experienced significantly greater increases in average weight gain and physical therapy index than patients treated with diet alone. This efficacy of oxandrolone in burn patients is not age dependent (61). Recently, oxandrolone (20 mg/d) administered during the immediate postburn period to patients with burns covering 40–70% of their body surface area produced a decrease in net weight loss, an increase in nitrogen retention, and a decrease in healing time compared with placebo (62). Furthermore, oxandrolone has an equal anabolic potential as human GH and is, in fact, safer (63). In summary, the use of oxandrolone in this patient population has shown positive results. Therefore, we recommend judicious use of AAS in patients who have major burn injuries and are severely catabolic.

Cancer. Anorexia and weight loss are common occurrences in patients with cancer. Cachexia is a state of increased resting energy expenditure that continues despite decreased host reserves. Furthermore, weight loss in cancer is different from that in starvation. During starvation, the body adapts to use fat as the major source of fuel while conserving protein. In cancer-associated wasting, weight loss ensues due to equal losses of protein and fat. Increased utilization of amino acids for gluconeogenesis is responsible for muscle catabolism (64). The cachexic/anorexic effects of cancer lead to malnutrition and contribute to androgen deficiency (65). Therefore, AAS may have a role to play in the treatment of cancer cachexia. However, only a few controlled trials have been performed to ascertain whether this represents an influence of hormones on nutritional intake or *vice versa*. Preclinical trials with nandrolone decanoate in rats did not support the former hypothesis (66). In 1988, Todd (65) reported that malnutrition and the resulting weight loss in patients with pancreatic cancer are responsible for hypogonadism, rather than hypogonadism being the culprit. In either case, depressed serum T could result in decreased anabolic activity and loss of LBM.

Androgen therapy may also have other benefits in patients with cancer. Patients with cancer are anemic due to either malnutrition or the effect of chronic disease. Androgen therapy results in increases in hemoglobin levels (1–5 g/dl) and red blood cell volume (325–350 ml) (67, 68). Before the availability of recombinant erythropoietin, refractory anemia, especially secondary to bone marrow failure, was treated successfully with androgen therapy (69).

The erythropoietic effects of androgens have been known for many years in patients with leukemia. Clinical studies show that the treatment with stanozolol during the induction phase of chemotherapy results in a positive effect on the duration of remission (70, 71). However, with the advent and wide availability of recombinant erythropoietin, androgen use has become rare. It may be appropriate in certain circumstances, however, when wasting accompanies anemia or when cost is an issue.

Renal failure. Malnutrition and sarcopenia are commonly seen in patients with end-stage renal disease receiving dialysis (72). As parenteral nutrition has proven to be ineffective in improving the nutritional status of these patients, AAS

therapy appears to be an exciting alternative. In a recent double blind, placebo-controlled trial, 29 patients were randomized to either placebo or nandrolone decanoate (100 mg/wk, im) for 6 months (73). Serum creatinine and LBM were significantly greater in the nandrolone group. The results of functional tests such as timed walking and stair-climbing also significantly improved in the nandrolone group, whereas they worsened in the placebo group.

In addition to the increase in LBM, patients with chronic renal failure benefit from the stimulation of erythropoiesis resulting from the administration of AAS (74, 75). A recent study of 25 male anemic patients with normal serum iron levels showed an increase in erythropoietin synthesis in 15 patients treated with nandrolone decanoate (200 mg/wk, im) for 6 months (76). Although erythropoietin levels returned to baseline 6 wk after the final dose of nandrolone, the hemoglobin concentration remained in the normal range up until 16 wk after discontinuation of nandrolone. Clinical trials have shown that nandrolone decanoate therapy in combination with recombinant human erythropoietin result in a greater increase in hematocrit compared with erythropoietin alone (77). Based on these positive data, the role of AAS should be further studied in patients with renal failure, especially evaluation of functional status and quality of life.

Safety

At this point little is known about the complications of AAS therapy in patients with cachexia. The safety information that is available is mainly from the use of AAS in athletes. A 1997 survey of 97 body builders using AAS reported various side effects, including testicular atrophy, gynecomastia, hypertension, fluid retention, tendon injuries, nosebleeds, frequent colds, hepatic and renal dysfunction, and sleep irregularities (78). Studies examining these effects are described below in further detail.

Effects on gonads. A reduction in fertility associated with anabolic steroid use results due to gonadotropin suppression, which, in turn, results in azoospermia, abnormalities in sperm motility and morphology, and testicular atrophy (79–81). The reversibility of these effects is variable. Some have suggested that restoration of hormonal balance after discontinuation of AAS use allows testicular function to return to normal (82, 83), whereas other studies have shown the persistence of hormonal abnormalities even after discontinuation of AAS (84, 85).

Muscular-skeletal injury. Despite the apparent positive effects of AAS on bone and muscle strength, alterations in connective tissue structure induced by AAS therapy have been associated with deleterious effects on tendon strength. Evidence suggests that anabolic steroid use leads to dysplasia of collagen fibrils, resulting in a decrease in overall tendon tensile strength (86). The risk of triceps tendon rupture, a relatively uncommon injury, is also increased in association with AAS use (87). Further research focusing on the risk of tendon injury in both athletic and nonathletic populations should be conducted.

Lipoproteins. Fluctuations in lipid profile are often seen in patients receiving AAS therapy. Palatini *et al.* (88) compared

10 body builders using AAS to 14 body builders who did not receive any anabolic agent. At the completion of the study, subjects taking AAS had lower high density lipoprotein (HDL) cholesterol and elevated low density lipoprotein concentrations. Similarly, weekly im administration of nandrolone decanoate (200 mg/wk) to 14 hemodialysis patients resulted in a significant decrease in HDL-2 cholesterol and apolipoprotein A-I levels (89). An increase in the concentrations of apolipoprotein B and triglycerides was also seen. The use of 17 β -esterified derivatives have less adverse effects on serum lipids than oral 17 α -alkylated analogs (90–92). Interestingly, T has been shown to be less deleterious to the lipid profile compared with other AAS. Thompson *et al.* (93) in their 6-wk cross-over trial of 11 male weight lifters showed that administration of oral stanozolol at a dose of 6 mg/d resulted in a more adverse lipid profile than im injection of supraphysiological dose of T (200 mg/wk). Serum HDL levels decreased by 33% during stanozolol treatment compared with a decline of 9% during T administration. The reason for this difference may be due to an increase in the activity of hepatic triglyceride lipase (the enzyme responsible for HDL catabolism) in response to oral agents (93). In the future, more comparative studies between T and other AAS concerning their effects on lipid profile would be helpful.

Cardiovascular. For more than 6 decades, T has been known to induce hypertension in animals (94). Animal studies have shown that AAS inhibit 11 β -hydroxylation of 11-deoxycorticosterone to corticosterone, which results in hypertension in rats (95). Fluid retention may also contribute to hypertension. The human heart expresses the AR and hence is a target organ for androgens (96). Cardiomegaly has been reported in the preclinical studies of AAS (97,98), and electron microscopy shows disintegration of intercalated discs, mitochondriolysis, myofibrilolysis, and intracellular edema when AAS is given in conjunction with physical training (99). The risk of atherosclerosis may also be increased with AAS use, as shown by an increase in aortic elastin and collagen content with T administration to male rats. A study of male athletes found significantly greater cardiovascular risk factors in AAS users than nonusers (100). Subjects using AAS had a high total cholesterol/HDL ratio, higher low density lipoprotein levels, and lower HDL levels compared with nonusers.

The incidence of cardiovascular morbidity associated with AAS use, however, has been difficult to determine, partially because of the clandestine nature of steroid use in athletes. In patients undergoing therapeutic AAS treatment, there were only 16 reported cases of morbid circulatory events between 1976 and 1993 (101). No clinical study has yet demonstrated a conclusive link between AAS use and fatal cardiovascular events. However, patients with COPD should be followed more carefully, because the use of AAS may aggravate their polycythemia, thus predisposing them to myocardial ischemia and congestive heart failure.

Hepatic. Studies have linked various abnormal liver function tests (elevated plasma alkaline phosphatase, aminotransferases, conjugated bilirubin, and plasma proteins) with the use of AAS (102–104). Jaundice occasionally occurs in patients with a previously normal functioning liver due to a hypersensitivity-type reaction. Cholestatic hepatitis has also

been reported with the use of 17 α -alkylated agents due to the accumulation of bile in the biliary canaliculi without any obstruction in the larger ducts (105). If jaundice occurs, it generally develops after 2–5 months of therapy. In the majority of the patients, elevation in transaminases is transient, with levels normalizing within a few weeks of discontinuation (106). In athletes, particular care should be exercised when interpreting liver function tests, because breakdown of skeletal muscle during intense training can result in elevation of transaminases (107). There has been some concern over the use of AAS in patients with AIDS, as many of these patients have subclinical hepatic disease. As protease inhibitors and oxandrolone are metabolized by cytochrome P450 3A4 enzyme system, combined use of these drugs may result in elevation of the oxandrolone concentration to harmful levels. We recommend further pharmacological study in this regard. Peliosis hepatis has also been reported with the use of AAS (108–111). Lastly, there have also been isolated reports of AAS use resulting in carcinomas of the liver (112–115). An exhaustive review of the literature by the authors failed to show any clear increase in the incidence of liver cancer associated with AAS use, and virtually all clinical studies of AAS-associated hepatoma have been isolated case reports. Furthermore, the majority of these patients were taking these compounds for approximately 1–7 yr. AAS may play a role in the development of hepatocellular hyperplasia and hepatocellular adenoma; however, these effects usually occur in patients taking high doses of AAS or untraditional combinations of 17 α -alkylated AAS (116, 117). Although *in vitro* analysis does show some evidence for altered liver function with the use of 17 α -alkylated steroids (118), therapeutic doses have not been decisively proven to cause hepatocellular carcinomas. Furthermore, in the isolated cases in which cancers were reported to develop subsequent to the use of AAS, detailed evaluation found them to be hyperplastic lesions that regressed upon withdrawal of the drug. Supporting this observation, a long-term study of patients treated with stanozolol or danazol and followed for 15–47 months did not show any harmful effect on the liver (119).

Psychiatric. The effects of T on human aggression are controversial. Anecdotal evidence supports the claim that anabolic steroid use results in a typical “roid rage” phenomenon, during which athletes experience an increase in aggression and irritability while using AAS. The validity of this assertion is questionable in consideration of the fact that virtually all evidence supporting this behavior is based on either case reports or correlational studies (120). A few well controlled studies have demonstrated an association between AAS use and feelings of aggression, alertness, irritability, anxiety, suspiciousness, and other mood extremes (121–123). However, these results have been contradicted by other studies that found no evidence of aggressive behavior even when supraphysiological doses of T were administered (124). Wang *et al.* (125) recently reported that T administration to hypogonadal men resulted in a significant decrease in anger, sadness, irritability, and nervousness along with an increased sense of well-being, energy, and friendliness.

Additional safety considerations

Traditionally, physicians have been concerned about the effects of T administration on the prostate. However, recent reviews suggest that the incidence of prostate cancer is not increased by T administration (126). Furthermore, there is no clear evidence that androgen administration results in the development of benign prostatic hypertrophy. Recently, Snyder *et al.* (127) completed the longest (3-yr) study of T administration to hypogonadal elderly men. During the study period, there was no significant difference in major prostate events between the T and the placebo groups; however, the androgen group did demonstrate a small increase in prostate-specific antigen levels. Furthermore, there was no difference between the two groups in urinary flow rate, urinary symptom score, or residual postvoid urine volume (127). Although these data suggest that T has a good safety profile, many more studies are required before a firm conclusion can be made. Androgen administration remains an absolute contraindication in patients with a history of prostate cancer.

Clinical applications

In this review we have attempted to make endocrinologists aware of the fact that AAS may have a wide range of use in clinical medicine. Although the basic chemistry of the compounds is well characterized, the absolute safety and efficacy of AAS use under any circumstance other than androgen replacement for male hypogonadism have remained in question. Conventional wisdom has dictated that the use of AAS is not warranted due to possible safety hazards. However, recent clinical studies investigating these issues more thoroughly are beginning to demonstrate a possible usefulness for AAS therapy. Although patient safety remains a primary concern, the devastating cachexic effects of disease states such as HIV/AIDS require specific treatment.

Currently, the AAS therapies used in the U.S. for the treatment of severe weight loss include T esters (200 mg im every 2 wk), oxymetholone (50–150 mg/d), and oxandrolone (20 mg/d). Adequate nutrition must always be the first line of therapy for weight loss. However, AAS may have a role in patients in whom nutrition and standard care have been ineffective. We think that there are sufficient positive data available to recommend AAS for the treatment of cachexia associated with the AIDS wasting syndrome. Although data from patients with burn injury-related cachexia are scant, the available data are positive, and therefore, we recommend judicious use of AAS in patients with major burns who are severely catabolic. We propose further research in exploring the role of AAS in the treatment of wasting due to renal failure, cancer, COPD, and postoperative recovery.

We suggest the following plan for the treatment of patients with cachexia associated with chronic diseases. As many of these chronic disease states are associated with hypogonadism, patients who are found to be hypogonadal should be started on physiological T replacement therapy with either a patch or im injection (until further studies are available, we do not recommend supraphysiological doses of T in these patients). However, if these patients are eugonadal (normal T) despite being catabolic, we do not recommend therapy with AAS at this time.

Even though recent evidence suggests that administration of AAS such as oxandrolone to eugonadal men results in an increase in LBM (44), we believe that more research is needed to further evaluate the role of AAS such as nandrolone and oxandrolone in eugonadal catabolic patients.

Because of possible side effects associated with AAS therapy, several precautions should be taken before administering AAS. The possibility of altered liver function, especially with 17 α -alkylated anabolic steroids, warrants serial liver function testing. The androgenic nature of all anabolic steroids necessitates the testing of PSA levels in men before therapy is initiated. Additionally, serum lipids should be checked, as AAS therapy may be detrimental to patients at high risk for cardiovascular complications, especially those with low serum HDL levels.

The goal of AAS therapy, along with appropriate nutrition, would be to increase weight and LBM, which would translate into an improvement in functional status and reductions in mortality. Unfortunately, the number of studies evaluating these outcomes are limited. Moreover, there is a great need to evaluate the role of AAS in women with wasting syndromes. Although at this time we can recommend AAS in a limited number of conditions, further research is needed on the use of AAS in multiple diseases and their impact on quality of life and survival.

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INTERACTION OF GLUCOCORTICOID ANALOGUES WITH THE HUMAN GLUCOCORTICOID RECEPTOR

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Summary—Transient co-transfection of receptor cDNA and suitable reporter genes was used to study human glucocorticoid receptor (hGR) function in a neutral mammalian cell background. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the hGR and to bind to extracts of cells expressing the hGR cDNA. There was very good correlation between these two *in vitro* parameters for these compounds. Furthermore, correlation of these data with reported *in vivo* anti-inflammatory potencies was surprisingly close, with two exceptions. The *in vitro* data suggest an explanation for the discrepant compounds, consistent with published data on their metabolic fate *in vivo*. The co-transfection assay has utility as a quantitative predictor of *in vivo* glucocorticoid pharmacology.

INTRODUCTION

The human glucocorticoid receptor (hGR), cloned in 1985 [1], is known to be a member of a protein superfamily of closely related intracellular receptors (IRs) which function as ligand-activated transcription factors [2-4]. The hormone-IR complex can positively or negatively regulate the expression of gene networks by its interaction with specific target hormone response elements (HREs) within the promoters of controlled genes. One useful development in the elucidation of the structure and function of the GR protein was the development of a "cis-trans" or co-transfection assay in which glucocorticoid-dependent transcriptional control could be reconstituted in a model cell system [5]. This has enabled significant advances in the understanding of the domain structure of the hGR and other IRs [5-7]. In the "cis-trans" assay, a plasmid encoding the cDNA for the hGR under a constitutive promoter, e.g. the Rous sarcoma virus (RSV) long terminal repeat (LTR), and a second plasmid carrying a gene for a detectable reporter, e.g. firefly luciferase (LUC), under the control of a glucocorticoid-responsive promoter, e.g. the LTR of the mouse mammary tumor virus

(MMTV), are introduced into a neutral mammalian cell background. This results in reconstitution of hormone-dependent transcriptional transactivation of reporter gene expression. This introduction of the hGR cDNA and the MMTV LTR-LUC can be accomplished by preparing suitable adenoviral vectors [9] or by transient transfection of two plasmids, one directing overexpression of hGR and the other encoding MMTV LTR-LUC. In addition to conferring on recipient CV-1 cells a functional and measurable response to glucocorticoids, adenoviral infection or transient transfection with the hGR-encoding vector also confers specific binding of tritiated dexamethasone.

The availability of a reliable and quantitative *in vitro* predictor of *in vivo* anti-inflammatory activity would have significant benefit. In the present study, we evaluate the utility of *in vitro* quantitative assessment of various glucocorticoid analogues using the "cis-trans" assay and radioligand binding as predictors of *in vivo* activity of these compounds. The potency and efficacy of a panel of 21 natural and synthetic glucocorticoids were assessed. The functional agonist activity of these 21 glucocorticoids was determined in CV-1 cells expressing transfected hGR cDNA over a full range of concentrations (10^{-11} to 10^{-5} M). This was compared to their potency to displace specifically bound tritiated dexamethasone from cytoplasmic extracts of CV-1 cells expressing the hGR protein after introduction of hGR cDNA.

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EXPERIMENTAL

Media and chemicals

CV-1 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Grand Island, NY) and supplemented with 2 mM L-glutamine (Gibco), and 55 μ g/ml gentamicin (Flow Laboratories, McLean, VA). Plasmids pRShGR, pGRE-LUC, and pRSV- β -gal have been previously described [5]. Briefly, pRShGR is a pBR322 derivative containing the hGR cDNA under control of the RSV LTR. pGRE-LUC contains a cDNA for LUC under the control of the MMTV LTR, a conditional promoter containing a glucocorticoid response element (GRE). pRSV- β -gal contains the gene for *E. coli* β -galactosidase under control of the RSV-LTR, a constitutive promoter. Unlabeled chemicals were obtained from Sigma Chemical Co. (St Louis, MO). [3 H]Dexamethasone (approx. 40 Ci/mmol) was purchased from Amersham Radiochemicals (Arlington Heights, IL).

Buffers and enzyme assays

For the competitive binding assay, homogenization buffer [(10 mM Tris-HCl (pH 7.4) 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM dithiothreitol, 0.25 M sucrose)] and gelatin phosphate buffer [0.15 M NaCl, 0.015 M NaN_3 , 0.1 M Na_2HPO_4 , 0.039 M NaH_2PO_4 (pH 7.0), 0.1% gelatin] were utilized.

Buffers utilized for the calcium phosphate mediated co-transfection assay were as described [8]. All cell washing steps were in 150 mM phosphate buffered saline (PBS). Test ligands were prepared in DMEM containing 10% (v/v) charcoal-absorbed fetal calf serum. Luciferase activity in cell extracts was assayed in 9.7 mM MgCl_2 , 1.66 mM ATP, 0.45 sodium luciferin, 91 mM potassium phosphate, pH 7.8 on a Dynatech luminometer as described [9]. Cell extracts were also analyzed for β -galactosidase activity as described previously [5].

Preparation of cell extracts

CV-1 cells were infected with Ad/MLUC7 [9], a recombinant adenovirus expressing the hGR cDNA, in the presence of dl309 helper virus (5 PFU/cell) by incubating cells and viruses in DMEM with 10% FBS (DMEM/FBS) at 37°C. After a 30 min adsorption, the mixture was diluted 10-fold with DMEM/FBS and plated in 15 cm culture dishes. At the end of an 18 h

incubation at 37°C, cells were detached and washed with PBS by centrifugation at 700 g for 5 min. All further procedures were carried out at 4°C. Cells were homogenized in 2 vol of homogenization buffer in a Teflon homogenizer with a motor-driven pestle at 1250 rpm. The homogenate was clarified by centrifugation at 1000 g for 15 min. A soluble cytosol fraction was generated by centrifuging this supernatant fraction at 104,000 g for 1 h. The protein concentration of the cytosol fraction was determined by dye binding [10], using bovine γ -globulin as standard. Cytosol fractions were used immediately or stored at -70°C.

Competitive binding assay

Aliquots of cytosol (100 μ g protein) were incubated at 4°C with 2.5 nM tritiated dexamethasone in the presence of incremental concentrations (0 – 2.5×10^{-5} M) of unlabeled dexamethasone or unlabeled cold competitor test compounds. After a 24 h incubation period at 4°C, unbound steroid was removed by addition of 2 vol of 7.5% dextran-coated charcoal in gelatin-phosphate buffer. The mixture was incubated for 10 min at 4°C and centrifuged at 3000 g for 10 min. The radioactivity in the supernatant fluid was determined by liquid scintillation counting. The non-specific binding was determined in the presence of unlabeled dexamethasone (10^{-5} M). All determinations were performed in duplicate.

Co-transfection assay

Co-transfections were performed essentially as described [5]. Sub-confluent CV-1 cells were passed at 3 day intervals to maintain good transfection efficiency. CV-1 cells were plated 24 h prior to transfection at 70% confluency. The recombinant DNA constructs were transiently transfected into CV-1 cells by calcium-phosphate co-precipitation [8]. Each plasmid preparation used for transfection was cesium banded twice prior to use. Following transfection, all subsequent steps were performed on the Biomek Beckman Automated Workstation. Medium was removed from transfected cells after 6 h, cells were washed and each glucocorticoid was tested at seven incremental concentrations in duplicate (10^{-11} – 10^{-5} M). After 38 h the cells were washed and lysed with 0.5% Triton-X 100 and assayed for luciferase and β -galactosidase activities, using a luminometer (Dynatech) and ELISA plate reader. The EC_{50} (concentration giving 50% of maximal observed

Table 1. Transactivation and binding of glucocorticoid analogues to hGR

Compound	Co-transfection		Binding	
	EC ₅₀	Normalized	IC ₅₀	Normalized
Dexamethasone	1.2E-09	0.04	3.8E-09	0.12
Fluocinolone	1.5E-10	0.00	1.5E-09	0.03
Betamethasone	8.1E-10	0.03	7.0E-09	0.14
Triamcinolone	5.3E-09	0.17	4.0E-08	0.80
6 α -Methyl prednisolone	1.3E-08	0.41	9.0E-09	0.18
Fludrocortisone	1.4E-08	0.44	1.3E-08	0.26
Prednisolone	2.7E-08	0.84	1.6E-08	0.32
Hydrocortisone	3.2E-08	1.00	5.0E-08	1.00
Corticosterone	4.7E-08	1.47	1.0E-07	2.00
Aldosterone	7.5E-08	2.34	7.2E-07	14.40
21-Deoxycortisol	2.5E-07	7.81	1.2E-07	2.40
11-Deoxycorticosterone	5.9E-07	18.44	5.0E-08	1.00
11-Deoxycortisol	7.6E-07	23.75	1.5E-07	3.00
Progesterone	2.5E-06	78.13	5.0E-08	1.00
Prednisone	> 1.0E-05		2.0E-06	40.00
Cortisone	> 1.0E-05		3.8E-06	76.00
Testosterone	> 1.0E-05		5.5E-06	110.00
Dihydrotestosterone			1.0E-05	200.00
Estriol	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisone	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisol	> 1.0E-05		> 1.0E-05	
Tetrahydrocorticosterone	> 1.0E-05		> 1.0E-05	
18-Hydroxydeoxycorticosterone	> 1.0E-05		> 1.0E-05	
18-Hydroxycorticosterone	> 1.0E-05		> 1.0E-05	

Note: normalization to hydrocortisone.

binding was displaced over approximately a two log concentration range. Representative data are shown in Fig. 1(B). Table 1 gives graphically determined concentrations required to inhibit 50% of specific binding of 2.5 nM [³H]dexamethasone (IC₅₀), both as absolute val-

ues (M) and normalized to the IC₅₀ of hydrocortisone. IC₅₀ values range over three logs, with fluocinolone the most potent compound and testosterone the least.

A comparison of normalized potency in the co-transfection assay and in the competitive

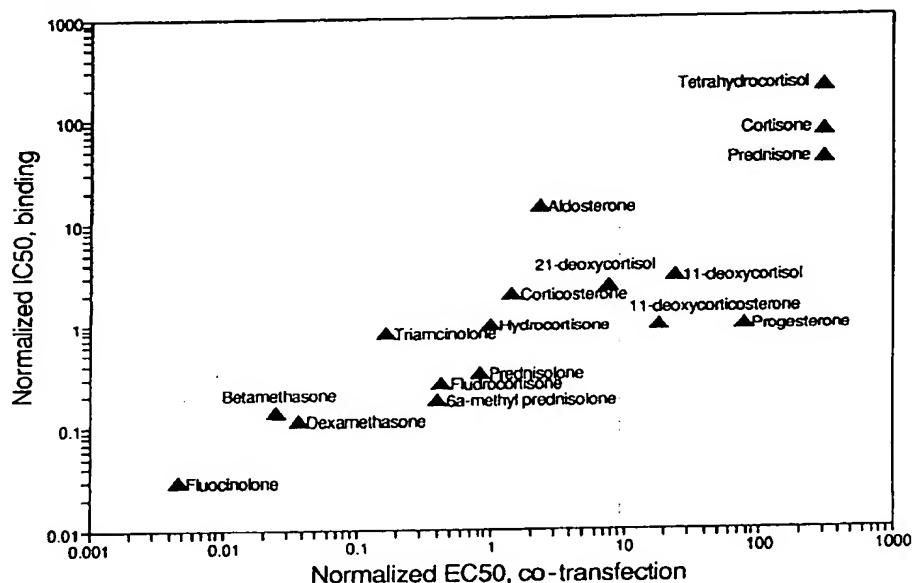


Fig. 2. Comparison of steroid binding and transactivation. Relative binding potency (ordinate) vs relative transactivation potency (abscissa). Competitive binding potency was determined for each analogue by measuring IC₅₀, i.e. the concentration required to inhibit by 50% specific binding of 2.5 nM [³H]dexamethasone to extracts of CV-1 cells over-expressing hGR cDNA as described (Experimental). Relative binding potency was derived by normalizing these data to the IC₅₀ of hydrocortisone. Transactivation potency was measured for each analogue in the co-transfection assay using hGR cDNA in CV-1 cells as described (Experimental). Data are expressed as EC₅₀, normalized to the EC₅₀ of hydrocortisone.

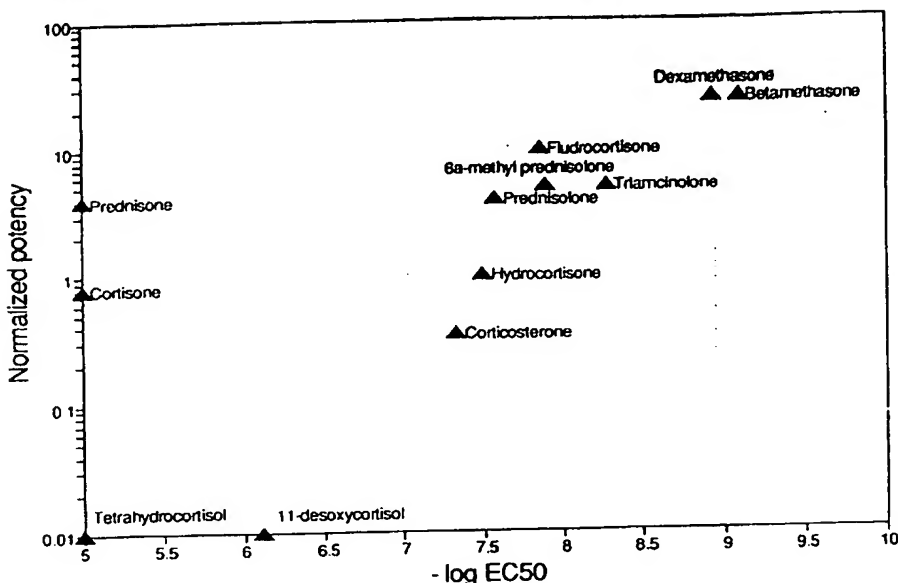
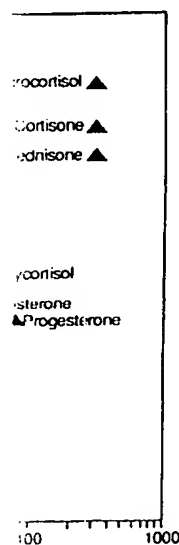


Fig. 3. Comparison of *in vivo* anti-inflammatory potency and *in vitro* hGR transactivation of glucocorticoid analogues. Relative anti-inflammatory potency *in vivo* (ordinate) vs *in vitro* transactivation potency (abscissa). Relative anti-inflammatory potency, normalized to that of hydrocortisone, is derived from reported values [11]. Transactivation potency was determined in the *in vitro* co-transfection assay using hGR cDNA introduced into CV-1 cells as described (Experimental). Values are expressed as $-\log EC_{50}$.

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(ordinate) vs relative each analogue by binding of 2.5 nM (Experimental). of hydrocortisone, using hGR cDNA d to the EC_{50} of

binding assay for the various analogues (Fig. 2) reveals an excellent correlation between these two parameters. A notable exception to this correlation is progesterone, which is equipotent with hydrocortisone in displacing [3H]dexamethasone, but about 80-fold weaker as a functional agonist of the hGR in the co-transfection assay.

Comparison of the biological potencies of the various steroids analyzed in the co-transfection assay *in vitro* compared to reported values of their *in vivo* anti-inflammatory potency ([11] based on dose in milligrams necessary for equal efficacy) is presented graphically in Fig. 3. Very good agreement is noted for all compounds, with the exception of prednisone and cortisone, which are much more potent *in vivo* than they are in the *in vitro* co-transfection assay in CV-1 cells.

DISCUSSION

The comparison of binding potency and transactivation agonist activity *in vitro* (Table 1 and Fig. 2) shows good correlation between the two for most of the 21 compounds examined. The partial agonist activity observed with fluocinolone (Fig. 1) does not appear to result from interaction of the compound with a

subset of the introduced hGR, since fluocinolone gives no agonist activity in CV-1 cells in the absence of transfected hGR cDNA and is capable of displacing 100% of the specifically bound [3H]dexamethasone from hGR-expressing CV-1 cell extracts. It is possible that the conformation of the fluocinolone-hGR complex is less effective than full agonists at interacting with other components of the transcriptional apparatus in CV-1 cells.

The present study underscores the feasibility of using the "cis-trans" assay for quantitative evaluation of potential hGR agonist. There are several significant theoretical advantages of the "cis-trans" assay over conventional radioligand binding assays. The most significant is that the assay determines not only whether a compound interacts with hGR but also the functional consequences of that interaction on gene expression, allowing the prediction of agonist and antagonist pharmacological effects. The assay, using hGR, can be expected to be less susceptible to potential species-related artifacts and inaccurate predictions than small-animal-based pharmacological studies. Finally, in screening for novel pharmacophores which might act as agonists or antagonists of the hGR, the "cis-trans" assay can detect any small molecules with functional consequences,

whether or not they interact with the receptor in the natural hormone binding site. Competitive radioligand displacement assays can only detect such compounds if their binding results in allosteric effects on the ligand site.

The agreement between the relative potencies of the 21 analogues as activators of hGR-dependent transcription and as competitors with dexamethasone binding was remarkably close (Table 1 and Fig. 2). There were a few compounds which showed greater ability to displace dexamethasone binding than ability to transactivate MMTV-LUC in CV-1 cells. Progesterone was equipotent with hydrocortisone in binding, but 80-fold less potent in transactivation. These data suggest that progesterone binds to the hGR but doesn't lead to the allosteric changes in hGR conformation necessary for transactivation. This failure could be at the level of dissociation of hGR and heat shock protein or at the level of interaction with the GRE. The data suggest that progesterone might antagonize glucocorticoid activation of the hGR.

In the case of the compounds for which estimates of *in vivo* anti-inflammatory potency were available, there was remarkably good correlation with the *in vitro* results in the "cis-trans" assay (Fig. 3). This reflects the extent to which the model cell system mirrors systemic sites of *in vivo* action. Two notable exceptions to this were prednisone and cortisone, both of which had low activity in the CV-1 cell assay. Formally, this could reflect either catabolism to inactive derivatives by CV-1 cells or the absence of necessary metabolic activation *in vitro*. The inactive compounds both have carbonyl functions at position 11. In the "cis-trans" assay, their 11-hydroxylated analogues, prednisolone and corticosterone, were about two logs more potent. Prednisone and cortisone were also relatively ineffective in displacing [³H]dexamethasone from hGR-containing extracts at 4°C *in vitro*, arguing against catabolism as the explanation of their lack of potency in the "cis-trans" assay. Prednisone and cortisone are known to require hepatic metabolic activation to prednisolone and corticosterone for *in vivo* activity [11]. This activating metabolic conversion apparently does not occur in CV-1 cells *in vitro*. With the

exception of these compounds requiring metabolic activation, the "cis-trans" assay is surprisingly predictive of *in vivo* potency.

In the present study, close correlation was found in most cases between the relative potencies of the 21 compounds in functional agonist activity *in vitro* and in radioligand displacement. Furthermore, there was excellent correlation between *in vitro* data and reported *in vivo* anti-inflammatory data for these compounds. Introduction by infection or co-transfection of hGR cDNA and a suitable reporter into a receptor-deficient mammalian cell establishes a hormone-inducible transcription system which be utilized to quantitate the pharmacological efficacy and potency of potential ligands for the human glucocorticoid receptor.

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Transdermal Dihydrotestosterone Treatment of 'Andropause'

Bruno de Lignieres

Male ageing coincides on average with progressive impairment of testicular function. The most striking plasma changes are an increase in sex hormone binding globulin (SHBG) and a decrease in non SHBG-bound testosterone, which is the only testosterone subfraction effectively bioavailable for target tissues. In healthy subjects the bioavailable testosterone declines by approximately 1% per year between 40 and 70 years but a more pronounced decline has been observed in non-healthy groups, especially in high cardiovascular risks groups. Relative androgen deficiency is likely to have unfavourable consequences on muscle, adipose tissue, bone, haematopoiesis, fibrinolysis, insulin sensitivity, central nervous system, mood and sexual function and might be treated by an appropriate androgen supplementation. The potential risk for prostate has been the main reason for limiting indications of such treatment. Testosterone (T) and dihydrotestosterone (DHT) are two potent androgens which have opposite effects regarding aromatase activity, an enzyme present in prostate stroma and suspected to have a pathogenic influence through local oestradiol synthesis. T is the main substrate for aromatase and oestradiol synthesis while DHT is not aromatizable and, at sufficient concentration, decreases T and oestradiol levels. A 1.8 years survey of 37 men aged 55–70 years treated with daily percutaneous DHT treatment suggested that high plasma levels of DHT (> 8.5 nmol/l) effectively induced clinical benefits while slightly but significantly reducing prostate size. Early stages of prostate hypertrophy require synergic stimulation by both DHT and oestradiol, and suppressing oestradiol instead of DHT seems easier and better adapted to the specific situation of aged hypogonadic men. Since the little information we have related to vascular risks also suggests that aromatase activity and oestradiol induce unfavourable effects, DHT may be considered an attractive alternative for long-term treatment of andropause.

Key words: male aging; secondary hypogonadism; dihydrotestosterone, prostate; aromatase.

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Introduction

Most investigators agree that male ageing coincides, on average, with progressive impairment of testicular function (1). Even if this phenomenon is not as consistent, abrupt and precisely age-related as menopause, the term 'andropause' is a close description of secondary gonadal insufficiency found in aged males. Vascular perfusion and Leydig cell numbers of testicular tissue

tend to decrease with age together with the ability to respond to LH stimulation and to produce physiological amounts of androgens. High amplitude LH pulses are also reduced in correlation with decreased LH-RH release in aged men (2).

Since the sex hormone binding globulin (SHBG), the main protein carrier of androgens in plasma, significantly increases with age, total plasma testosterone levels show only limited decrease. However, significantly less unbound testosterone, the only form effectively bioavailable for target tissue, circulates in the plasma, resulting in less actual androgenic stimulation (3–5).

There is great interindividual variability in the magnitude of testicular function impairment and the decline in plasma androgens with age. In a group of healthy men, excluding drinkers, those with obesity, and those with

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any known chronic illness or using any prescribed medication, the bioavailable testosterone (not bound to SHBG) is reported to decline by approximately 1% per year, then to drop nearly 30% between 40 and 70 years (3). However, a more pronounced age-related decline, in comparison with healthy subjects, has been observed in an unselected population (2) and related to specific characteristics such as overweight (6), abdominal obesity (7), insulin resistance (8), low HDL-cholesterol levels (9), low apoprotein A₁ levels (10), low fibrinolytic activity (11), high coronary artery stenosis score (12), low bone density (13), high incidence of any stressful events including any unspecific illness (14), as well as depressive mood, low incidence of nocturnal, penile tumescence (15) and reduced sexual activity (16). Plasma levels of oestradiol and dihydrotestosterone, two steroids resulting mainly from peripheral metabolism of testosterone, did not change significantly with age in a careful selection of healthy men (3). Strong binding of these two steroids to SHBG makes their plasma values difficult to interpret (17). Urinary excretion of 5 α -reduced metabolites is clearly reduced, suggesting a relative defect in 5 α -reductase (18). The most significant alteration of testosterone metabolism has been described in unselected population or in specific subgroups (3). An increased testosterone aromatization to oestradiol, in coincidence with a decreased reduction to dihydrotestosterone and then androstenediol, has been reported in obese subjects or in subjects exposed to various cardiovascular risks (12, 19–23). This situation is likely to reflect an impaired metabolism of androgens by target tissue with age which may worsen the consequences of the deficient bioavailable testosterone levels.

Therefore, age-related impairments in androgenic stimulation seem more pronounced in non-healthy groups, specifically in high cardiovascular risk groups, than in ideally healthy subjects. Since a statistically normal population of aged men includes high percentages of several non-healthy groups, there is increasing interest in androgen substitution (2). Androgens are the main anabolic hormones, and a chronic decrease in their bioavailability, contrasting with the sustained catabolic efficacy of cortisol (3), is likely to have a deleterious effect on muscle and bone. Androgens are also expected to have favourable consequences on bone marrow and haematopoiesis, the central nervous system and mood, and on sexual function (15, 24). However, both experimental studies and previous human experience suggest that the efficacy/safety ratio concerning the cardiovascular system and prostate may vary greatly according to the molecule, dose and method of administration of an androgenic treatment.

Androgen Formulations

More pharmacokinetic variability and metabolic side-effects than benefits are expected from the liver first pass effect of androgens, so parenteral administration is preferable to oral formulation for long-term replacement

(1). Since the most popular testosterone ester formulations injected at 3–4 week intervals result in huge intra- and interindividual variability in testosterone plasma values, alternative methods that produce constant androgen levels would be welcome (1). They would be easier to monitor on clinical or plasma value end-points and would reproduce a more consistent physiological androgenic stimulation. A gel formulation delivering androgen through the skin, once a day, has been available in several European countries since 1980 (25). Either testosterone (T) or dihydrotestosterone (DHT), the two main natural androgens, can be delivered by such a percutaneous formulation with similar clinical benefits in the short term (26, 27). Significant improvements in mood and sexual function can be elicited in hypogonadic men by increasing plasma levels of either testosterone or dihydrotestosterone. However, side-effects on the cardiovascular system and prostate differ according to the steroid administered, since T and DHT have different metabolisms in the target tissues. When T is administered to a hypogonadic man, a substantial amount of the androgen is aromatized to oestrogen, and oestradiol plasma values increase significantly from an average of 70% after cutaneous application (27) to more than 100% after intramuscular injection (28), reaching female follicular phase levels in many users. In contrast, when the non-aromatizable DHT is administered, mean DHT plasma values increase to 12–31 nmol/l according to the dose while testosterone levels decrease to less than 5 nmol/l, reversing the usual testosterone/dihydrotestosterone levels and, as a consequence, circulating levels of oestrogens tend to decrease (27, 29). Therefore, in comparison with T treatment which, besides androgenic stimulation, provides more circulating oestradiol and more substrate for intra-tissular synthesis of oestrogens, DHT treatment reduces circulating oestradiol and drastically reduces the androgenic substrate for aromatase activity, which may influence pathogenesis in some specific tissues such as the prostate and cardiovascular system.

Testosterone, Dihydrotestosterone and Cardiovascular Risks

Most studies report relatively low testosterone levels according to age, associated with several cardiovascular risks such as abdominal obesity, insulin resistance, low HDL cholesterol levels, low apoprotein A₁, low fibrinolytic activity and severe coronary artery stenosis (6–12). Treatment with parenteral administration of normal doses of either T or DHT to healthy aged men induced similar limited changes in circulating lipids with a tendency to lower total levels of cholesterol, triglycerides and LDL cholesterol, but also of apolipoprotein A₁ and HDL-cholesterol (27–29). Such complex plasma lipid changes, when induced by exogenous sex steroids, are now known to be poor predictors of atherosclerosis or cardiovascular risks. In contrast to the well known consequences of HDL cholesterol variations inversely related to triglycerides, the exact consequences of

parallel changes in HDL cholesterol and triglycerides remain speculative (30). For example, a dramatic reduction of both HDL cholesterol and triglyceride plasma levels may not induce atherosclerosis progression in females treated by androgenic progestins (31), but high oestrogen may increase cardiovascular risks in men despite their ability to increase both HDL cholesterol and triglyceride levels (32-35). Therefore, no clear conclusion can be drawn from the analysis of circulating lipoproteins in isolation, and we still lack experimental evaluation of atherosclerosis plaques in males with or without androgen treatments.

Parenteral administration of T tends to reduce body fat and increase lean body mass (36, 37). While huge doses may reduce insulin sensitivity (38), normal doses improve insulin sensitivity in middle-aged abdominally obese men (36). Similar human studies with appropriate doses of DHT are lacking, but experimental studies show that the muscle is sensitive to DHT as well as T (39), and that fat tissue is also reduced by DHT (40). Anabolic steroids, T and DHT treatments at normal doses increase fibrinolytic activity (41-43). Experimental studies show that very high T and DHT concentrations (10-100 times relative human doses) may increase platelet aggregation (44-46). In contrast to the recent information accumulated on various effects of physiological concentrations of oestradiol in females (47), little is known about any potential direct effects of normal doses of T and DHT on arterial wall metabolism and vasomotion in males. One study has ascribed significant reduction in exercise-induced electrocardiographic abnormalities in men, by parenteral administration of normal doses of T (48) which may be explained by coronary relaxation. Experimental studies in male rats also suggest that very high concentrations of testosterone may induce alterations of fibrous proteins, elastin and collagen content of the arterial wall (49).

A relative increase in oestradiol plasma levels does not seem favourable for men during acute myocardial infarct (50) and seems to be associated with more severe coronary stenosis (12, 21, 22), decreased apoprotein A1/B ratio and HDL cholesterol, increased triglycerides (12, 22), and higher incidence of overweight and tobacco consumption (19, 23). Massive administration of oestrogen in men dramatically increased the incidence of vascular accidents (32-35) despite apparently favourable changes in HDL and LDL cholesterol. This increase in risk seems to be related mainly to oestrogen-induced changes in both coagulation and fibrinolytic factors (35). On the other hand, nothing is known about the potential cardiovascular benefits of any drug-induced decrease in plasma and tissue oestradiol levels in men.

To summarize, men with moderate age-related hypogonadism seem to be at a higher risk of cardiovascular accidents than eugonadic men of the same age. Parenteral administration of either T or DHT in moderate doses, increasing plasma values to a mean of less than 10 ng/ml, did not induce significant clinical or metabolic side-effects in normal men (25-29, 36, 51).

According to recent investigations of the effect of oestrogen on atherosclerosis processes and coronary

ischaemia in females, it is obvious that prediction of vascular risks related to sex hormones cannot be adequately supported by isolated evaluation of plasma lipoproteins, combining apparent benefits (tendency to lower total and LDL cholesterol and to lower triglycerides) with apparent side-effects (tendency to lower HDL cholesterol and apoprotein A1). The few studies focusing on haemostatic factors conclude consistently that the effects of treatments by either T or DHT are beneficial. The existing literature does not support any significant increase in vascular risk in mid-term users of current doses of parenteral T or DHT. On the contrary, these treatments are likely to improve lean body mass, insulin sensitivity and fibrinolysis in men with impaired testicular function (36, 43). Very high doses are suspected of impairing insulin sensitivity, increasing platelet aggregation, thrombogenic risks and fibrous protein content of arterial wall, so the classical 3-4 weeks' injection schedule inducing very large plasma T peaks and valleys may be less than optimal for vascular protection and may not represent a convenient model of hormone replacement. A shorter cycle of injections with lower T doses (28) or daily cutaneous applications (25-27) are preferable for metabolic studies. Since a further increase in plasma and tissue estradiol concentration can hardly be considered beneficial in aged men with vascular risks, DHT may be an alternative long-term treatment for such patients.

Testosterone, Dihydrotestosterone and Prostate Risks

Prostate cancer and benign prostatic hypertrophy (BPH) are partly androgen-dependent diseases with a striking increase in incidence in aged men, so they are the major areas of concern for men receiving any kind of androgen for 'andropause' treatment (1). At least some degree of BPH, in comparison with normal young adults, becomes almost statistically normal in untreated men of 60 years or older (52) and it is crucial to understand whether androgenic treatment brings any risk of benefit to this situation. The first period of prostate growth is clearly related to puberty and testicular secretion of androgen, but the hormonal influences on the second period of prostate growth after 50 years are far from being precisely identified.

A second burst in testicular androgen secretion is clearly excluded; on the contrary, plasma levels of bio-available androgens tend to decrease after 50 years. DHT accumulation within the prostate tissue has been proposed as a plausible explanation (53) but was rapidly ruled out by most recent studies comparing prostate tissues with or without BPH from similar origins (54-55). Normal or even low levels of T or DHT in plasma and prostate tissues seem compatible with BPH, and limited regression in prostate volume is described only when an almost complete suppression, far below physiological levels of circulating T (56, 57) or tissue DHT (58, 59) is achieved by several pharmacological agents. More than 80% decline in DHT prostate tissue concentration by

5 α -reductase inhibitor induced a mean decrease of only 18% in prostate size (58, 59), and suppression of plasma testosterone levels to a mean of 0.3 nmol/l (50 times lower than physiological levels) is required to induce a 30–40% regression in prostate size (56, 57). These results do not support the concept that any spontaneous increase in T or DHT stimulation of stromal or epithelial prostatic cells may be an actual *primum movens* for BPH in aged man. Neither do they provide useful information about the risks and benefits for the prostate of a moderate compensatory increase in androgen stimulation in aged men.

Since no detectable supra-physiological increase in T and DHT stimulation of prostate can explain BPH, several other explanations have been proposed. One of them considers the age-related changing balance between oestrogens and androgens (60–63). Experiment studies consistently show the inability of androgens non-aromatizable to oestrogens to induce the early stage of prostate hypertrophy (64, 65). Aromatizable androgens such as T and androstenedione may induce hyperplastic changes in the prostate of monkeys but these effects are reversed by addition of an aromatase inhibitor (66).

In the same way, treatment with non-aromatizable DHT of male L.W. rats susceptible to cancer of the prostate has not induced gross or microscopic prostate hypertrophy nor prostate cancer after 14 months of exposure in any animals. By contrast, rats treated with similar doses of testosterone showed an increased incidence of benign hypertrophy in the stroma; adenocarcinoma were also identified in 24% of treated animals with additional *in-situ* tumours in 16%. Among the untreated control group an intermediate situation was observed, with some cases of benign hypertrophy and some *in situ* neoplasms but no invasive adenocarcinoma (65). Such an experimental study suggests that in animals with high prostate disease risk, T and DHT have quite different effects, long-term aromatizable T exposure increasing incidence of both benign and malignant prostate diseases, while exposure to non-aromatizable DHT decreases both risks. A short-term study with an aromatase inhibitor in men with BPH has reported a 26% reduction in mean prostate size (67), supporting the concept of at least some oestrogen dependence in humans.

There is a good rational basis for such an oestrogen effect on the prostate. Oestradiol receptors have been identified in normal human prostate or in early stage of BPH, with higher concentrations in stromal than in epithelial cells (68). Aromatase activity, which locally synthesizes oestrogens from substrates like testosterone or androstenedione, has been identified in the stromal part of the prostate (69). Moderate concentrations of oestrogens in the presence of androgens stimulate specifically stromal growth in the short term (70), but in the mid and long term stromal cells influence epithelial growth through paracrine effects (71–73) while androgens, specifically DHT, influence primarily epithelial growth and maintain a physiological balance between stroma and the epithelium (70). It is not only aromatase activity which tends to increase with age (74), some experimental studies also suggest a slight tendency for

less sensitivity of prostate cells to DHT with age with, on the other hand, more sensitivity to oestradiol (70). Following this theory a progressive imbalance between oestradiol and DHT may appear in the prostate tissue of aged men. Oestradiol stimulation may show a relative increase following the rise in oestrogen plasma levels, tissue aromatization of testosterone, and potential receptivity of stromal cells due to autocrine/paracrine changes. Firstly, stroma will proliferate, creating growing nodules which will influence, secondly, glandular elements through non-androgenic stimulating paracrine process. Thirdly, autocrine/paracrine factors will keep both stromal and epithelial cells under control, the most aggressively growing part of the tissue becoming largely independent of any steroid stimulation.

To summarize, according to this pathogenic explanation, neither DHT without oestradiol nor oestradiol without DHT would overstimulate stromal or epithelial prostatic cells (75). The *primum movens* for prostate changes in middle-aged men is probably a progressive increase in oestradiol/DHT ratio within the stroma. Until today most hormonal manipulations have been directed to androgen suppression and have produced objective benefits only when androgen stimulation has been reduced to almost zero level. This situation, created either by oestrogens in sufficiently high doses to suppress gonadotrophins or by LH-RH agonists or, to a lesser extent, by 5 α -reductase inhibitors, does not respond directly to the suspected pathological process and obviously does not respond to the need for an 'andropause' hormonal substitution. The anti-androgenic treatment may suppress more normal androgen-dependent cells than oestrogen-stimulated cells potentially harmful in the long term. Their benefits may be limited in duration. On the other hand, DHT treatment which, unlike T, includes both androgenic and anti-aromatase activity, is an attractive possibility; in theory, it increases androgenic stimulation while preventing, or drastically reducing the age-related tendency to an increasing oestradiol/DHT ratio. Reduction of oestrogens, unlike reduction of androgens, has no visible side-effects in ageing men.

A third theory considers the striking age-related SHBG rise as the best pathogenic explanation for prostate diseases. The SHBG excess, not carrying a high affinity steroid ligand, is able to bind to some membrane receptors identified in prostate cells (76) and then to stimulate growth through an autocrine effect. The growth of human carcinoma cells *in vitro* is stimulated by addition of SHBG to the culture media (77). Further addition of T to the media does not change or enhance the SHBG effect. By contrast, addition of high concentrations of DHT decreases the stimulating effect of SHBG. Saturating plasma SHBG by high affinity ligand such as DHT prevents SHBG binding to cell membrane receptor (77). This third theory is not inconsistent with the second one, because SHBG rise is likely to be related to an age-related increase of aromatase activity in the liver, and because it also suggests potential benefit for DHT use in comparison with T. Finally, instability in androgen stimulation may be of pathogenic importance since intermittent androgenic stimulation of the prostate stimulates

more mitosis in prostate epithelium than does a constant stable concentration of androgen (78). This suggests that long-acting testosterone formulations with large oscillations in plasma androgens may be less than optimal for *in vivo* human prostate studies.

Very few studies have investigated prostate changes in ageing men during androgen supplementation. Thirteen men, 57–70 years old, with relatively low non-SHBG-bound T plasma values but normal prostate size (according to ultrasonographic and prostate-specific antigen evaluation) have been treated by T enanthate injections (100 mg per week) for 3 months. During such a short treatment period the mean prostate-specific antigen level has increased significantly and has remained elevated 3 months after stopping the treatment. This limited result suggests that T supplementation may create prostatic side-effects in older men (28).

We have investigated prostate changes in 37 men, 55–70 years old, with high SHBG plasma levels and clinical symptoms attributed to hypogonadism during a 6 months to 5 years duration of percutaneous DHT treatment. In 27 subjects DHT plasma levels have been increased up to 8.5–20 nmol/l. As a consequence, gonadotrophins have decreased, T plasma levels have been reduced to less than 5 nmol/l (1.7–4.8) and oestradiol plasma levels have dropped by 50%. In this group, prostate size has been significantly reduced according to both ultrasonographic and PSA evaluation. Mean prostate size was 31.09 ± 16.31 g before treatment and 26.34 ± 12.72 (–15.4%) after a mean 1.8 year of DHT treatment ($P=0.01$).

In contrast, in 10 men with lower plasma DHT levels (<8.5 nmol/l), a smaller decrease in T plasma values (>7 nmol/l) and no change in oestradiol plasma values, a slight non-significant enlargement of prostate size has been observed. Mean prostate size was 31.6 ± 16.38 g before treatment and 36.15 ± 16.62 g, 1.7 year later (+14.4%) in this group.

A larger cohort study comparing prostate size evolution in DHT treated and untreated men of similar age and initial prostate size is presently in progress.

Conclusion

Ageing males may suffer from an age-related secondary hypogonadism related to testicular and hypothalamic impairment. The most striking plasma changes are an increase in SHBG and a decrease in non-SHBG-bound T, which is the only T effectively bioavailable for target tissues. This kind of 'andropause' seems more frequent in non-healthy subjects, not only those with sexual dysfunction but also those with several cardiovascular and bone risks. Like menopause in females, 'andropause' might be treated by an appropriate androgen supplementation in some specific subgroups of ageing males. Potential risks for the prostate are the main reasons for limiting the indication for and duration of androgenic treatments. Benign prostate hyperplasia or prostate adenocarcinoma may progress even with normal or significantly reduced androgenic stimulation in comparison with young healthy adults, and only an almost com-

plete early suppression of total androgen offers effective protection against prostate disease. Therefore, there is no realistic expectation for a prostate protective effect when prescribing testosterone supplementation, even at physiological doses. However, some drug-induced qualitative changes in T metabolism to DHT by 5 α -reductase or to oestradiol by aromatase may influence prostate tissue. Since the first hypothesis to explain the burst in prostate diseases incidence after 50 years has focused on a possible DHT accumulation in prostate tissue, 5 α -reductase inhibitors have recently been proposed. However, the clinical benefits for prostate seem limited and they are not useful alone for any 'andropause' treatment. They have not yet been used in addition to a testosterone treatment. Since the DHT accumulation hypothesis is now denied, the potential responsibility of oestradiol, through an age-related increase aromatase activity is now under consideration and has been supported by several *in vitro* and experimental studies. DHT and T seem equally effective for inducing the expected benefits of mood, sexual function, bone and muscle. However, they have opposite effects regarding aromatase. T is the main substrate for synthesis of oestradiol while DHT is not aromatizable, and, at sufficiently active concentration, decreases T and oestradiol levels. BPH induction requires both DHT and oestradiol, and suppressing oestradiol instead of DHT seems easier and better adapted to the specific situation of aged hypogonadic men. Preliminary human studies suggest that androgen supplementation is safer for the prostate of ageing men when they are treated by DHT instead of T. Since the little information we have related to vascular risks also suggests that aromatase activity and oestradiol have unfavourable effects, DHT may be considered an attractive alternative for long-term treatment of andropause.

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The Effect of a Pure Antiandrogen Receptor Blocker, Flutamide, on the Lipid Profile in the Polycystic Ovary Syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies affecting women of reproductive age; it is associated with hyperandrogenism, hyperinsulinemia, and dyslipidemia. This study was designed to assess the long term effects of a pure androgen receptor blocker, flutamide, on the lipid profile in women with PCOS and to examine the possible mechanisms by which androgens may exert their influence. Seventeen women with PCOS (10 obese and 7 lean) were studied. All subjects received a 12-week course of oral flutamide (500 mg/day). The baseline and posttreatment evaluations included lipid profile, androgen levels, insulin sensitivity, and serum catecholamine determinations. The primary outcome was the change in the ratio of low density lipoproteins (LDL) to high density lipoproteins (HDL). Treatment with flutamide was associated with a signif-

icant decrease in the LDL/HDL ratio by 23% ($P = 0.005$), in total cholesterol by 18% ($P < 0.0001$), in LDL by 13% ($P = 0.002$), and in triglycerides by 23% ($P = 0.002$). Flutamide treatment was also associated with a trend toward an increase in HDL (by 14%; $P = 0.14$). The effects on lipid profile were found regardless of obesity and were not associated with a change in weight. Furthermore, actions of flutamide on lipid metabolism were not associated with significant changes in circulating adrenaline or noradrenaline, glucose metabolism, or insulin sensitivity. This report has demonstrated for the first time that treatment with the pure antiandrogen, flutamide, may improve the lipid profile and that this effect may be due to direct inhibition of androgenic actions. (*J Clin Endocrinol Metab* 83: 2699–2705, 1998)

POLYCYSTIC ovary syndrome (PCOS) is one of the most common endocrinopathies, affecting 5–10% of premenopausal women (1). Hyperandrogenism in women with PCOS is associated with metabolic aberrations of much greater importance than cosmetic problems such as hirsutism or acne. These aberrations include insulin resistance with accompanying hyperinsulinemia and dyslipidemia (2–8). To our knowledge, there is no definitive prospective study demonstrating that PCOS represents an independent of obesity risk factor for development of cardiovascular disease. However, several retrospective and case-control studies indicated that, in the long term, women with a history of PCOS may be at increased risk for developing hypertension, noninsulin-dependent diabetes mellitus, atherosclerosis, coronary artery disease, and myocardial infarction (6, 9–11).

Most reports suggest that the lipid profile of women with PCOS is characterized by elevated serum levels of cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL), and triglycerides, with concomitantly reduced concentration of high density lipoproteins (HDL) (5, 6, 8, 12–15). These abnormalities in lipid levels have serious atherogenic consequences; in particular, as observed in the

Framingham study, high LDL and low HDL predict the development of coronary artery disease (16, 17).

Dyslipidemia typically occurs within a cluster of several interrelated cardiovascular risk factors, including obesity, high waist to hip ratio (WHR), hyperinsulinemia, and hyperandrogenism. The elucidation of cause and effect relationships among these factors and hence the identification of independent risk factors are exceedingly difficult. In light of recent studies, it appears that although obesity is a major contributor to dyslipidemia in women with PCOS, some abnormalities of lipid profile are independent of obesity. Graf *et al.* observed that obesity was associated with a decrease in HDL levels in both PCOS and control subjects (18). Subsequently, Robinson *et al.* observed that low HDL in women with PCOS cannot be explained solely by obesity, indicating, therefore, that other inherent features of PCOS predispose to dyslipidemia (19).

Potential mechanisms of dyslipidemia in women with PCOS include hyperinsulinemia and hyperandrogenism. Alternatively, genetic variation in each of these mechanisms modified by environment may be occurring. The roles of insulin and sex hormones in the regulation of lipid metabolism have been well recognized. In particular, levels of HDL₂ are regulated by lipoprotein lipase and hepatic lipase; these activities are responsive to insulin and sex steroids, respectively (20, 21). Ek *et al.* observed that women with PCOS had an impairment of catecholamine-induced adipocyte lipolysis due to defects such as a decreased number of

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β_2 -adrenoreceptors as well as postreceptor dysfunction of the protein kinase A or hormone-sensitive lipase (22). These defects in the adrenergic regulation of lipolysis may be attributable to insulin resistance and hyperinsulinemia.

Elevation of androgens offers another plausible explanation of metabolic disturbances in insulin resistance syndromes such as PCOS (23, 24). Hyperandrogenism is associated with upper body obesity (expressed as WHR) independently of weight (25–27). Furthermore, administration of exogenous androgen to women leads to increased visceral fat accumulation and decreased serum HDL (28). However, the mechanisms of androgen actions on lipid metabolism are still poorly understood. There is growing evidence that androgens may influence the predominant site of body fat deposition and muscle morphology, possibly in relation to alterations in splanchnic insulin metabolism and insulin sensitivity (29–32). It appears that androgens may also adversely affect lipid metabolism by direct modulation of lipoprotein lipase and lipolysis (33). The effects of androgens on lipid profile may be sex dependent; studies in hypogonadal men are inconsistent and indicate that androgen replacement may have either an adverse or a beneficial effect (34, 35).

The present study was undertaken to assess the long term effects of a pure androgen receptor blocker, flutamide, on the lipid profile of women with PCOS and to examine the possible mechanisms by which androgens may exert their influence. To our knowledge, this is the first report demonstrating that flutamide exerts a beneficial effect on serum lipids.

Subjects and Methods

Subjects

Seventeen women with PCOS (10 obese and 7 lean) were studied (Table 1). The diagnosis of PCOS was made in the presence of chronic anovulation, and hyperandrogenism was confirmed by an elevation of at least 2 of the following plasma androgens: total testosterone, free testosterone, androstenedione, and 3 α -androstenediol glucuronide. Hirsutism and acne were present in 9 subjects, 6 subjects had only hirsutism, and 2 subjects had only acne. The diagnosis of PCOS was further substantiated by the finding of thickened stroma and multiple subcapsular cysts on ovarian ultrasonographic examination.

Subjects with diabetes mellitus, adrenal disorders, hyperprolactinemia, or other endocrine disorders were excluded. Congenital adrenal hyperplasia was ruled out by evaluation of 17-hydroxyprogesterone and, when appropriate, ACTH stimulation test. None of the patients had hypertension or overt heart disease. The subjects were not taking any drugs known to affect gonadal function or carbohydrate metabolism.

TABLE 1. Profiles of obese and lean participants in the study

	Obese (n = 10) ^a	Lean (n = 7)
Age	22.7 \pm 1.0	22.0 \pm 1.7
Wt (kg) ^b		
Pretreatment	81.6 \pm 4.6	55.3 \pm 3.3
Posttreatment	81.5 \pm 4.6	56.0 \pm 3.1
Ht (m)	1.63 \pm 0.02	1.62 \pm 0.03
BMI (kg/m ²)	30.6 \pm 1.3	20.9 \pm 0.8
WHR	0.89 \pm 0.03	0.81 \pm 0.04

Results are presented as the mean \pm SEM.

^a Subjects were considered obese when their BMI was at least 25 kg/m².

^b Weight posttreatment was not significantly different from baseline weight.

Body mass index (BMI) was calculated as weight (kilograms)/height (meters)². Patients were considered lean when their BMI was below 25 kg/m² and obese when their BMI was at least 25 kg/m². Body fat distribution was assessed by measuring the WHR as described by others (36).

After the completion of the baseline studies (see below), all subjects received a 12-week course of oral flutamide (Flucinom, Schering-Plough, Kenilworth, NJ) at a dose of 500 mg/day. Flutamide treatment was initiated on the first day of the menstrual cycle. The patients were advised not to change their eating habits or their activities throughout the study period. All participants were nonsmokers and nondrinkers; they received regular meals with a weight-maintaining diet. The study was approved by the local ethics board. Informed consent was obtained from all patients. Throughout the study, the subjects were using barrier contraception.

Study protocol

All evaluations were conducted within 10 days from the onset of menstrual flow. In the absence of spontaneous menstruation, periods were induced by medroxyprogesterone acetate withdrawal. The baseline evaluations were performed as follows: blood samples were collected at 0800 h after an overnight fast to determine serum levels of steroids (testosterone, free testosterone, androstenedione, and 3 α -androstenediol glucuronide), sex hormone-binding globulin (SHBG), and lipids (total cholesterol, triglycerides, HDL, and LDL). Glucose metabolism and insulin sensitivity were evaluated using an oral glucose tolerance test (OGTT) and hyperinsulinemic-euglycemic clamp procedure. OGTT was performed by collection of baseline blood samples followed by ingestion of a 75-g glucose load and subsequent blood sampling at 30-min intervals for 2 h to determine serum levels of insulin and glucose; the test was used to determine the area under the curve (AUC) for glucose and insulin. Insulin sensitivity was determined (5–7 days after OGTT) by measuring glucose uptake during the hyperinsulinemic euglycemic clamp procedure, as described previously (23). Briefly, after an overnight fast, catheters were inserted into a dorsal hand vein and an antecubital vein. Crystalline human insulin (Actrapid, Novo-Nordisk, Athens, Greece) was infused via a Harvard pump (Harvard Apparatus, Millis, MA) at a rate of 287 pmol (40 mU)/m²·min for 180 min to increase the plasma insulin level to approximately 500 pmol/L (\sim 75 μ U/mL). Serum glucose was kept constant at the fasting level with the aid of bedside serum glucose determinations every 5 min and appropriate adjustment of a variable infusion of 20% glucose. Serum adrenaline and noradrenaline were assessed at a baseline (from three pooled samples collected every 5 min) and at the end of the hyperinsulinemic-euglycemic clamp study (from pooled samples collected at 170, 175, and 180 min). All of the above studies were repeated at the end of the 12-week course of flutamide.

Analytical procedures

Plasma glucose was determined with a Beckman glucose analyzer (Palo Alto, CA), using a glucose oxidase method. A solid phase ¹²⁵I RIA was used for quantitative measurement of serum insulin levels as described by others (29). Serum samples were analyzed using commercial RIA kits to determine levels of total testosterone (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and free testosterone (Diagnostic Products Corp., Los Angeles, CA). Androstenedione, dehydroepiandrosterone sulfate (DHEAS), and 3 α -androstenediol glucuronide were measured using kits from Diagnostic Systems Laboratories (Webster, TX). For all determinations, the intra- and interassay coefficients of variation were 5–7% and 8–11%, respectively. Total cholesterol, triglycerides, and HDL were determined using techniques of Spectrum FIPX (Abbott Laboratories, North Chicago, IL), whereas LDL was calculated by the Friedewald equation. Adrenaline and noradrenaline were determined using kits from DDN Diagnostika (Mirpur, Germany).

Statistical analysis

The values were expressed as the mean \pm SEM. Comparison of means was performed using Student's *t* test or ANOVA, as appropriate. Baseline and posttreatment levels of lipids and hormones were compared using repeat measures ANOVA with one grouping factor (obese *vs.*

lean). When appropriate, log transformation of variables was performed.

Results

Baseline and posttreatment levels of lipids

Treatment with flutamide resulted in marked changes in the lipid profile. Comparisons of lipid levels before treatment (baseline) and after the 12-week course of flutamide (post-treatment) are presented in Table 2. The results were analyzed using repeated measures ANOVA; this approach allows simultaneous evaluation of the effects of treatment, obesity, and the role of interaction between treatment and obesity. In the context of this study, the interaction component may be interpreted as a determination of whether the effect of treatment was altered by obesity. Flutamide treatment was associated with an average decline in cholesterol by 18%, in triglycerides by 23%, and in LDL by 13%. Treatment with flutamide was also associated with a trend (albeit not statistically significant) toward an increase in HDL by 14%. Ultimately, the LDL/HDL ratio declined by 23%. Effects of obesity on lipid profile were not statistically significant; nevertheless, there was a trend among obese patients to have higher levels of triglycerides and LDL. Comparable effects of flutamide were observed in both obese and lean patients.

The effects of a 12-week course of flutamide on lipid profile in individual patients are presented in Fig. 1. Flutamide treatment was associated with a decline in the cholesterol level in all subjects. Triglycerides decreased in 9 of 10 obese patients and in all 7 lean patients. LDL declined in 7 of 10 obese patients and in 6 of 7 lean patients. HDL increased in 6 of 10 obese patients and in 6 of 7 lean patients.

Baseline and posttreatment endocrine profiles

Table 3 presents comparisons of baseline and posttreatment levels of individual endocrine parameters. Treatment with flutamide was associated with an average decline in 3α -androstenediol glucuronide by 21%, in androstenedione by 48%, and in DHEAS by 37%. Flutamide treatment had no significant effect on the level of total or free testosterone or estradiol. SHBG declined during flutamide treatment, on the average, by 18%. Flutamide had no demonstrable effect on levels of adrenaline and noradrenaline. Furthermore, flut-

amide treatment had no significant effect on fasting glucose levels, glucose AUC in response to oral glucose load, fasting insulin, insulin AUC in response to the glucose load, and glucose uptake during hyperinsulinemic, euglycemic clamp studies. Power analysis revealed that the present study had sufficient power (type II error <0.2 for type I error <0.05) to detect a 30% change in catecholamine levels or insulin sensitivity.

Obese women had, on the average, 61% higher free testosterone levels than lean subjects. There was no significant difference in the levels of 3α -androstenediol glucuronide, androstenedione, DHEAS, total testosterone, estradiol, or SHBG between obese and lean women. Furthermore, obese and lean subjects had comparable levels of adrenaline and noradrenaline. Obesity had no significant effect on fasting glucose and glucose AUC. However, obese women, compared to lean subjects, had higher fasting insulin (by 155%), higher insulin AUC (by 46%), and lower glucose uptake (by 31%) as determined by hyperinsulinemic, euglycemic clamp studies. It should be noted that due to the relatively small number of subjects, this study had limited power to evaluate the effects of obesity. Patients were categorized as lean or obese to better assess the effects of flutamide while accounting for a potentially confounding role of obesity. There was no significant interaction between the effects of treatment and obesity on any of the studied endocrine parameters. This observation indicates that flutamide treatment resulted in comparable effects in both obese and lean subjects. Flutamide treatment had no significant effect on the ovulatory status of the subjects; serum progesterone levels remained in the non-ovulatory range.

Hepatic function

Serum levels of hepatic enzymes were evaluated before and at the end of the treatment period (Table 4). Flutamide treatment was associated with modest increases in the results of liver function tests; however, these changes were not clinically significant.

Discussion

The results of this study indicate that 1) flutamide treatment is associated with a significant reduction in total

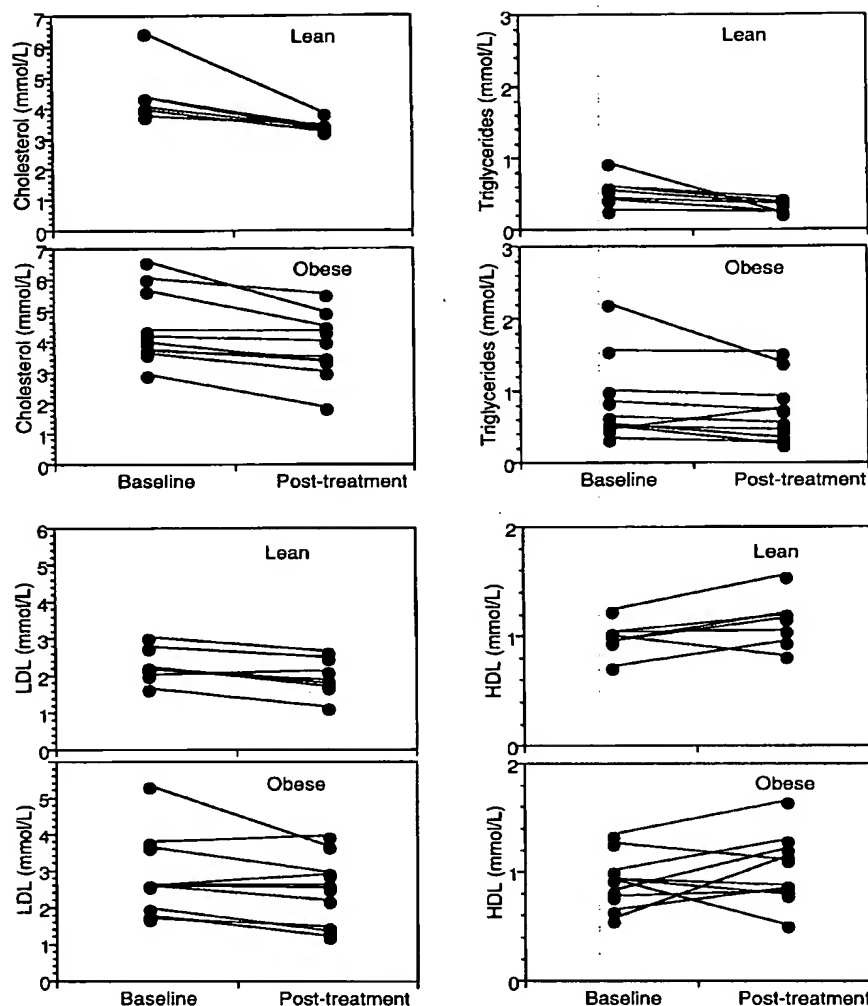
TABLE 2. Baseline and posttreatment lipid levels

Variable	Obese		Lean		P value ^a		
	Baseline	Posttreatment	Baseline	Posttreatment	Effect of treatment	Effect of obesity	Interaction: treatment vs. obesity
Cholesterol (mmol/L)	4.50 \pm 0.38	3.85 \pm 0.33	4.49 \pm 0.35	3.43 \pm 0.08	<0.0001	0.64	0.20
Triglycerides (mmol/L)	0.88 \pm 0.19	0.73 \pm 0.14	0.56 \pm 0.08	0.33 \pm 0.03	0.002	0.05	0.10
HDL (mmol/L)	0.92 \pm 0.08	1.03 \pm 0.10	1.00 \pm 0.06	1.14 \pm 0.09	0.14	0.82	0.81
LDL (mmol/L)	2.87 \pm 0.35	2.50 \pm 0.30	2.31 \pm 0.17	1.99 \pm 0.19	0.002	0.27	0.88
LDL:HDL ratio	3.46 \pm 0.29	2.66 \pm 0.39	2.35 \pm 0.19	1.79 \pm 0.19	0.005	0.18	0.78

Results are presented as the mean \pm SEM. Lipid levels are presented in Systeme International units. Normal ranges are: cholesterol, less than 5.2 mmol/L (multiply by 38.7 to convert to milligrams per dL); triglycerides, less than 1.8 mmol/L (multiply by 88.6 to convert to milligrams per dL); LDL, less than 3.36 mmol/L (multiply by 38.7 to convert to milligrams per dL); HDL, more than 1.29 mmol/L (multiply by 38.7 to convert to milligrams/dL).

^a Comparison of baseline and posttreatment levels determined using repeated measures ANOVA with one grouping factor (obese vs. lean). Interaction component of statistical analysis may be interpreted as a determination of whether the effect of treatment was different among obese vs. lean patients.

FIG. 1. Effect of a 12-week treatment with flutamide on lipid profile in lean and obese women with PCOS. Each line joins baseline and posttreatment values for each individual patient. Lipid levels are presented in Systeme International units. Normal ranges are: cholesterol, less than 5.2 mmol/L (multiply by 38.7 to convert to milligrams per dL); triglycerides, less than 1.8 mmol/L (multiply by 88.6 to convert to milligrams per dL); LDL, less than 3.36 mmol/L (multiply by 38.7 to convert to milligrams per dL); and HDL, more than 1.29 mmol/L (multiply by 38.7 to convert to milligrams per dL).



cholesterol, LDL, and triglycerides in young women with PCOS; 2) these effects occur in both obese and lean subjects; 3) the greatest effects of flutamide may be found in those with highest baseline levels of SHBG and androstenedione; and 4) the actions of flutamide on lipid metabolism appear not to be related to changes in circulating adrenaline and noradrenaline levels, glucose metabolism, or insulin sensitivity.

The foremost importance of the present findings is the potential for the development of new therapeutic strategies for the treatment of dyslipidemia. The effects of flutamide on lipid levels are consistent with a significant decline in the risk for development of atherosclerosis and consequent cardiovascular disease.

Most of the subjects in the present study did not have overt dyslipidemia. The potential therapeutic value of flutamide in the treatment of dyslipidemia has yet to be assessed in a broad population of subjects, over a longer treatment period, and with careful evaluation of possible adverse side-effects.

Although significant complications due to flutamide use are uncommon, patients may develop elevations of liver transaminases; furthermore, isolated cases of cholestatic hepatitis and even liver failure have been documented in elderly patients treated for prostatic cancer (37). Furthermore, a case of serious hepatotoxicity was reported in a woman treated with flutamide for hirsutism (38). In this study, flutamide produced no significant side-effects.

Flutamide is considered to be a "pure" androgen antagonist, acting by competitive inhibition of androgen receptors (39). Therefore, its actions on lipid profile may be most likely attributed to direct blockage of androgenic effects. As androgens are known to promote dyslipidemia, it is reasonable to expect that flutamide may promote a favorable lipid status by inhibiting these adverse actions of androgens. This line of reasoning is also supported by the findings of regression analysis, which indicate that the greatest improvement of total cholesterol may be found in patients with the highest pretreatment levels of androstenedione. In other words, the

TABLE 3. Baseline and posttreatment endocrine profiles

Variable	Obese		Lean		Effect of treatment	P value ^a	
	Baseline	Posttreatment	Baseline	Posttreatment		Effect of obesity	Interaction: treatment vs. obesity
3 α -Androstanediol glucuronide (nmol/L)	18.5 \pm 3.1	15.1 \pm 3.1	15.5 \pm 3.5	11.6 \pm 1.9	0.047	0.43	0.88
Androstenedione (nmol/L)	18.3 \pm 2.9	10.4 \pm 1.0	18.3 \pm 4.9	8.4 \pm 0.9	0.006	0.74	0.72
DHEAS (μ mol/L)	11.1 \pm 2.0	7.1 \pm 1.0	10.6 \pm 2.1	6.4 \pm 1.6	0.02	0.76	0.94
Free testosterone (pmol/L)	16.7 \pm 1.8	14.8 \pm 2.2	10.8 \pm 1.0	9.3 \pm 1.7	0.36	0.02	0.81
Total testosterone (nmol/L)	3.8 \pm 0.8	3.6 \pm 0.7	2.1 \pm 0.3	2.4 \pm 0.6	0.90	0.12	0.62
Estradiol (pmol/L)	279 \pm 44	262 \pm 56	306 \pm 52	310 \pm 97	0.92	0.54	0.88
SHBG (nmol/L)	57.9 \pm 12	45.2 \pm 9	88.0 \pm 22.4	75.3 \pm 15.5	0.04	0.15	0.99
Adrenaline, 0 min (ng/L)	30.8 \pm 1.2	31.0 \pm 1.3	32.4 \pm 3.3	37.9 \pm 9	0.09	0.19	0.10
Adrenaline, 180 min (ng/L)	31.6 \pm 1.8	30.6 \pm 1.3	32.1 \pm 2.2	38.5 \pm 5.1	0.15	0.21	0.06
Noradrenaline, 0 min (ng/L)	106.6 \pm 10.7	94.0 \pm 8.3	118.5 \pm 19.2	110.4 \pm 12.5	0.27	0.37	0.81
Noradrenaline, 180 min (ng/L)	102.3 \pm 9.0	97.9 \pm 9.4	96.0 \pm 10.6	99.1 \pm 9.7	0.88	0.85	0.40
Fasting glucose (mmol/L)	5.0 \pm 0.2	5.0 \pm 0.2	5.1 \pm 0.4	4.7 \pm 0.2	0.41	0.59	0.41
Glucose: AUC ^b	14.8 \pm 0.8	15.0 \pm 0.8	15.6 \pm 0.9	13.1 \pm 1.0	0.13	0.59	0.09
Fasting insulin (mU/L)	20.2 \pm 3.7	19.3 \pm 3.1	7.9 \pm 0.3	9.4 \pm 0.5	0.76	0.02	0.18
Insulin: AUC ^b	150 \pm 13	158 \pm 10	103 \pm 19	112 \pm 14	0.21	0.03	0.95
Glucose uptake (mmol/kg·min) ^c	254 \pm 21	271 \pm 26	369 \pm 42	383 \pm 39	0.16	0.02	0.87

Results are presented as the mean \pm SEM.

^a Comparison of baseline and posttreatment levels determined using repeated measures ANOVA with one grouping factor (obese vs. lean). Interaction component of statistical analysis may be interpreted as a determination whether the effect of treatment was different among obese vs. lean patients.

^b The area under the curve (AUC) for glucose and insulin was determined during a 2-h oral glucose tolerance test (OGTT).

^c Glucose uptake (moles per L) determined during euglycemic, hyperinsulinemic clamp studies represents a measure of insulin sensitivity.

TABLE 4. Liver function tests before and at the end of flutamide treatment

Test	Range	Median	Mean \pm SEM
Aspartate aminotransferase (normal range, 5–40 IU/L)			
Baseline	8–19	13	13 \pm 1
End of treatment	7–69	15	22 \pm 4
Alanine aminotransferase (normal range, 5–40 IU/L)			
Baseline	7–46	13	15 \pm 2
End of treatment	6–87	13	24 \pm 6
γ -Glutamyl transpeptidase (normal range, 10–40 IU/L)			
Baseline	4–48	8	14 \pm 3
End of treatment	3–68	9	17 \pm 4
Alkaline phosphatase (normal range, 30–125 IU/L)			
Baseline	30–67	45	45 \pm 3
End of treatment	10–82	48	49 \pm 5

greatest improvement may be expected in those with the greatest initial androgenic effect.

Lipid metabolism may be affected by various interlinked and interdependent mechanisms (18, 40). Thus, androgens may affect lipids not only directly, but also by affecting obesity, catecholamines, and insulin. In this study, treatment with flutamide had little or no effect on weight; furthermore, the improvement of lipid profile was observed in both obese and lean patients and regardless of the WHR. Consequently, although obesity and high WHR are important risk factors for dyslipidemia, the actions of flutamide cannot be attributed to the effects on total body fat and its distribution. Treatment with flutamide also had no significant effect on

adrenaline or noradrenaline; thus, it is unlikely that flutamide may have acted via modulation of metabolism of catecholamines. Furthermore, flutamide had no effect on fasting or post-OGTT insulin levels and glucose uptake during euglycemic clamp studies. Consequently, the effects of flutamide cannot be explained by the alterations in insulin sensitivity and its levels. Interestingly, Lovejoy *et al.* observed that administration of exogenous androgen to women led to increased visceral fat accumulation and decreased serum HDL without a change in fasting glucose or insulin sensitivity (28). Thus, androgens may affect lipid metabolism and fat deposition by mechanisms not involving insulin.

Actions of flutamide on lipid metabolism may not be universal to all pure antiandrogens. Casodex, another nonsteroidal agent that blocks androgen receptors, had no significant effect on total cholesterol, HDL, or LDL in men (41). In other studies on men, the use of cyproterone acetate, a synthetic steroid antiandrogen, resulted in adverse changes in the lipid profile, most notably a decrease in HDL (42, 43). These effects of cyproterone acetate may be due to its progestogenic properties.

Although flutamide is considered a pure antiandrogen, there is evidence that it has other biological activities and may, for example, inhibit adrenal 17–20-lyase (44). This activity may explain our present observation that flutamide treatment resulted in decreases in 3 α -androstanediol glucuronide, androstenedione, and DHEAS.

The present study revealed a complex and unexpected interrelationship between flutamide and SHBG. Treatment with flutamide led to a decrease in SHBG levels in both lean and obese patients; furthermore, high baseline SHBG level has been identified as one of the predictors of a greater decline in the total cholesterol level. Previous reports have demonstrated that hepatic production of SHBG may be in-

hibited by insulin and androgens (45, 46). Yet in this study, flutamide had no detectable effect on insulin. Furthermore, antiandrogenic properties of flutamide would be expected to increase, rather than decrease, the SHBG level. Thus, an observed decline in SHBG may be due to flutamide acting via mechanisms independent of its antiandrogenic properties. Another explanation of the suppression of SHBG involves the possibility that some actions of androgens may be independent of androgen receptors and thus not be inhibited by flutamide. Indeed, Brown *et al.* observed that testosterone may act on the liver by a mechanism independent of androgen receptors (47). In this context, it is important to note that although flutamide blocks androgen receptor-mediated effects, it has no significant effect on the levels of free and total testosterone. Thus, it is possible that in the presence of flutamide, testosterone may maintain suppressive activity on hepatic SHBG production. This explanation may also apply to the study by Winneker *et al.*, who found that flutamide failed to increase SHBG after its suppression by androgen treatment (48).

The present study was limited to evaluation of a single dose of flutamide and did not include a placebo group. The dose of flutamide was selected on the basis of previous studies demonstrating its effectiveness in the treatment of hyperandrogenic conditions such as hirsutism, acne, and hair loss (23, 49, 50). Ideally, the effects of flutamide on lipid profile should be reevaluated in a placebo-controlled trial assessing several doses of the drug.

In conclusion, this report has demonstrated for the first time that treatment with the pure antiandrogen, flutamide, may improve the lipid profile. The beneficial actions of flutamide appear to be independent of obesity, catecholamine metabolism, and insulin resistance.

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- [54] **TREATMENT OF PROSTATE DISEASE WITH A NONSTEROIDAL ANTI-ANDROGENIC COMPOUND**
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- [73] Assignee: **Merck & Co., Inc.**, Rahway, N.J.
- [21] Appl. No.: **806,944**
- [22] Filed: **Feb. 26, 1997**

Related U.S. Application Data

- [60] Provisional application No. 60/012,638, Mar. 1, 1996.
- [51] Int. Cl.⁶ **A61K 31/16; G01N 33/574; C12N 5/00**
- [52] U.S. Cl. **514/563; 435/7.23; 435/325; 435/352; 435/384**
- [58] Field of Search **514/563; 435/7.23, 435/325, 352, 384**

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Primary Examiner—David M. Naff

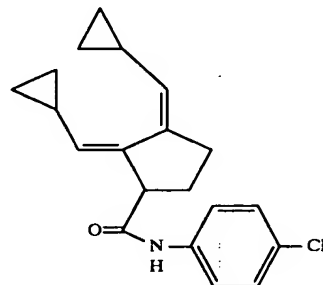
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[57]

ABSTRACT

An assay for identifying compounds with antiandrogenic activity employing a hamster ductus deferens cell line (DDT1) ATCC CRL-1701 and ATCC CRL-12051 is disclosed, as well as antiandrogenic compounds identified from this assay, particularly the nonsteroidal compound N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene) cyclopentane carboxamide of structural formula (I):



(I)

This compound is an antiandrogen useful in the treatment and prevention of diseases of the prostate including prostatitis, benign prostatic hyperplasia (BPH) and prostatic carcinoma.

4 Claims, 2 Drawing Sheets

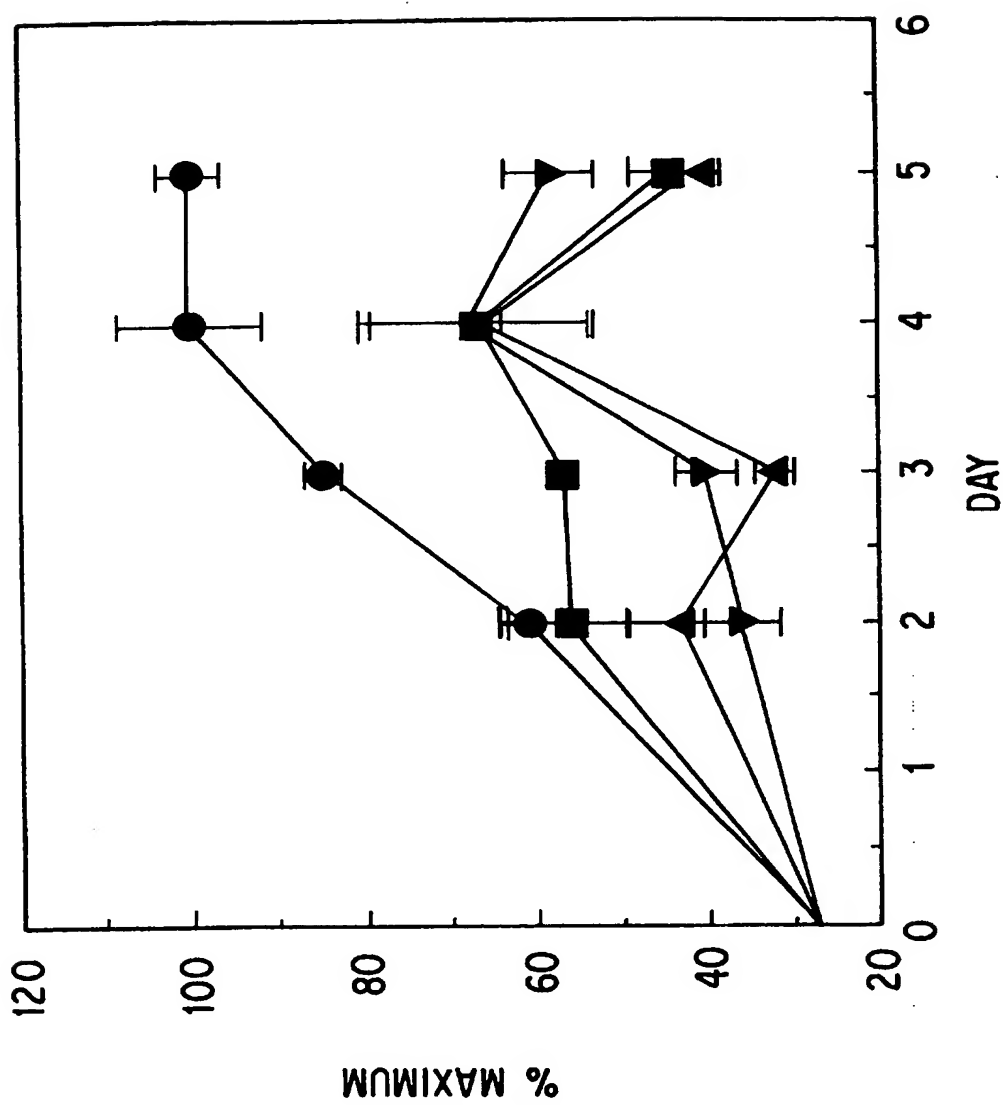


FIG.1

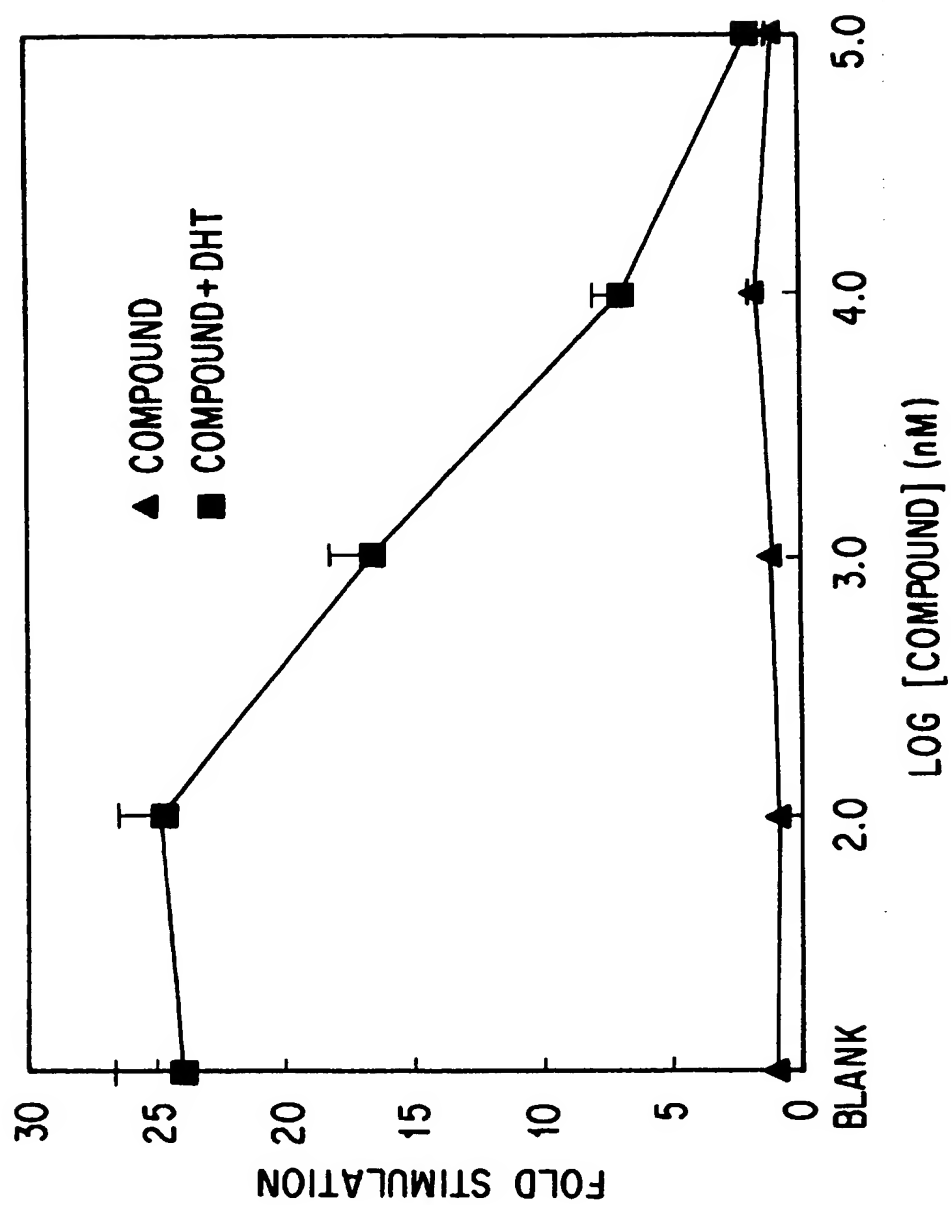


FIG. 2

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TREATMENT OF PROSTATE DISEASE WITH A NONSTEROIDAL ANTI- ANDROGENIC COMPOUND

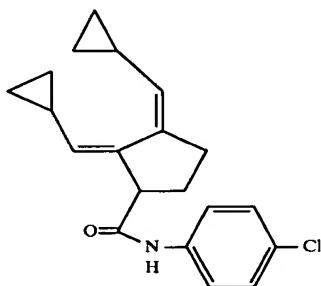
CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority of provisional application 60/012,638, filed Mar. 1, 1996.

SUMMARY OF THE INVENTION

The present invention relates generally to a novel assay for identifying compounds with antiandrogenic activity as well as to antiandrogenic compounds identified from this assay. The assay employs a hamster ductus deferens cell line (DDT1) ATCC CRL-1701 and ATCC CRL-12051 that is highly dependent on the addition of testosterone in synthetic serum-free media.

The nonsteroidal compound N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide of structural formula (I):



has been identified using the assay of the present invention as an antiandrogenic compound useful in the treatment and prevention of diseases of the prostate including prostatitis, benign prostatic hyperplasia (BPH) and prostatic carcinoma.

The present invention also provides for novel compositions employing compounds which exhibit antiandrogenic activity in the assay of the present invention. The compounds which exhibit antiandrogenic activity in the assay of the present invention are useful in the systemic, including oral, and parenteral, including topical, treatment and prevention of diseases of the prostate including prostatic carcinoma, benign prostatic hyperplasia and prostatitis.

The compounds, including N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide, identified as androgen antagonists in the assay of the present invention may be used in combination with other active agents, for example, a 5 α -reductase inhibitor such as finasteride, epristeride, 17 β -N-(2,5-bis(trifluoromethyl))phenylcarbamoyl-4-aza-5 α -androst-1-en-3-one, or the compounds described in PCT publication WO 95/11254, or an α 1- or α 1 $_a$ -adrenergic receptor antagonist, or combinations of such other active agents with the androgen antagonist compound identified from the present assay, wherein such combinations would be useful in one or more of the above-mentioned methods of treatment or pharmaceutical compositions.

BACKGROUND OF THE INVENTION

The DDT1 cell line has been reported to be an androgen-responsive cell line. Norris et al. "Androgen Receptors in a Syrian Hamster Ductus Deferens Tumour Cell Line," *Nature* 248:422-424 (1974). Syms et al., "Glucocorticoid Effects on Growth, and Androgen Receptor Concentrations on

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DDT₁MF-2 Cell Lines", *J. Steroid Biochem.*, 28(2):109-116 (1987) report that the DDT₁-MF-2 smooth muscle tumor cell line contains receptors for and is differentially sensitive to androgens and glucocorticoids. They report that androgens stimulate growth, and glucocorticoids inhibit growth of the cell line.

Harris et al., "Androgens and Glucocorticoids Modulate Heparin-Binding Growth Factor I mRNA Accumulation in DDT1 Cells as Analyzed by in situ Hybridization", *Mol. Endo.* 3(11):1839-1844 (1989), describe the ductus deferens smooth muscle tumor cell line (DDT₁-MF-2) as steroid-sensitive. They report that treatment with 10 nM testosterone accelerated the growth of DDT1 cells in the absence of serum, and that glucocorticoids inhibit growth. They further identify the DDT₁-MF-2 cell line as a useful model for steroid responsive tumor growth in vitro.

There has been a need for tissue culture models that mimic the androgen responsiveness observed in androgen sensitive diseases, particularly that observed in human prostatic carcinomas. The present invention provides for an assay to study compounds as potential anti-androgens in living cells.

(i) Lippman et al. in "The Effects of Androgens and Antandrogens on Hormone-response Human Breast Cancer in Long-Term Tissue Culture" *Cancer Research* 30: 4610-4618 (1976), describe the MCF-7 androgen-responsive cell line. Unlike the cell line of the present invention, the MCF-7 cell line cannot be grown in synthetic media.

Similarly, Marugo et al. in "Effects of Dihydrotestosterone and Hydroxyflutamide on Androgen Receptors in Cultured Human Breast Cancer Cells (EVSA-T)" *J. Steroid Biochem. Mole. Biol.* 42(5):547-554 (1992), describe another androgen responsive cell line. However, like the cells of the MCF-7 cell line, these cells cannot be grown in synthetic media.

The human androgen receptor has been isolated and characterized.

Tilley et al., "Characterization and Expression of a cDNA Encoding the Human Androgen Receptor", *Proc. Nat'l. Acad. Sci. USA*, 80:327-331 (1989) report the isolation, characterization and expression of the cDNA encoding the human androgen receptor, which predicts a protein of 917 amino acids and a molecular weight of 98918.

Rasmusson et al., "Therapeutic Control of Androgen Action", *Ann. Rep. Med. Chem.* 29:225-234 (1994) present a review of the androgen receptor, chemical antagonists of the androgen receptor, and means of controlling androgen biosynthesis.

Compounds identified as anti-androgens by the assay of the present invention are especially useful in the prevention and treatment of prostatic carcinoma, and they may also be useful in the treatment and prevention of other hyperandrogenic diseases such as acne vulgaris, seborrhea, female hirsutism, androgenetic alopecia, also called androgenic alopecia, which includes male and female pattern baldness, and benign prostatic hyperplasia.

Benign prostatic hyperplasia (BPH) and prostatic carcinoma are among the most common afflictions of aging men.

Benign prostatic hyperplasia is often treated surgically with a procedure known as transurethral resection of the prostate (TURP). Other surgical procedures performed to release the obstruction of urine include incision or stents. Castration has also resulted in regression of prostatic enlargement. Drug therapy for BPH has included alpha-1 blockers which treat the symptoms of the disease by alle-

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viating obstructive symptoms, but do not affect the underlying cause of the disease, the enlarged prostate gland. Representative alpha-1 blockers used in the treatment of BPH include: prazosin, terazosin, doxazosin, tamsulosin and alfuzosin. These drugs relax prostatic smooth muscle tone, decreasing intraurethral pressure without affecting bladder pressure. Common side effects of these agents are dizziness, headache and fatigue.

Finasteride (17 β -(N-tert-butylcarbonyl)-4-aza-5 α -androst-1-ene-3-one), which is marketed by Merck & Co., Inc., under the tradename PRQSCAR®, is an inhibitor of testosterone 5 α -reductase currently marketed for the treatment of benign prostatic hyperplasia. A principal mediator of androgenic activity in the prostate is 5 α -dihydrotestosterone ("DHT"), formed locally in the prostate by the action of testosterone-5 α -reductase. Inhibitors of testosterone-5 α -reductase inhibit the conversion of testosterone (T) to DHT and serve to prevent or lessen symptoms of hyperandrogenic stimulation in the prostate. See especially U.S. Pat. No. 4,377,584 assigned to Merck & Co., Inc., issued Mar. 22, 1983. The utility of finasteride in the treatment of prostatic carcinoma is also disclosed in the following documents: EP 0 285,382, published 5 Oct. 1988; EP 0 285 383, published 5 Oct. 1988; Canadian Patent no. 1,302,277; and Canadian Patent no. 1,302,276.

Both prostatic carcinoma and BPH have been treated with antiandrogens. Nonsteroidal antiandrogens such as flutamide and Casodex compete with DHT for androgen receptor sites in the prostate cells. These non-steroidal antiandrogens do not substantially change sexual potency and libido as the gonadotrophin releasing hormone agonists and progestogens do; however, these nonsteroidal antiandrogens often exhibit the undesirable tendency to feminize the male host (gynaecomastia) or initiate feed-back effects which would cause hyperstimulation of the testes.

Gonadotrophin-releasing hormone (GnRH) agonists such as nafarelin, busarelin, goserelin and leuporelin all reduce the release of leutinizing hormone (LH) by desensitizing the GnRH receptors in the anterior pituitary gland. GnRH agonists are able to reduce the production of testosterone, induce shrinkage of prostate volume and reduce the severity of urinary symptoms of BPH. Unfortunately, these drugs have adverse effects such as impotence and flushing, which discourage a majority of patients from continuing with the drugs. These androgen-suppressing agents are thus of inconsequential significance in BPH treatment, but are of major importance in the treatment of patients with advanced prostatic cancer.

Progestogens, such as megestrol acetate, hydroxyprogesterone and medrogestone depress testosterone by inhibiting LH release and blocking androgen receptors, causing a reduction in prostatic volume. Adverse effects such as decreased libido and impotence have kept progestogens from common use in BPH treatment.

Thus, there still remains a need for additional therapies for BPH and prostatic carcinoma for individuals who cannot tolerate the side effects and/or do not experience adequate relief from presently available therapies.

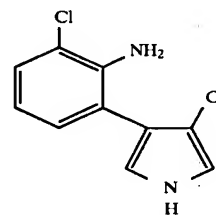
There also remains a need for a compound for the treatment of diseases of the prostate that is a non-steroidal compound having different pharmacological properties from steroids.

Hori et al. "WB2838 [3-Chloro-4-(2-amino-3-chlorophenyl)-pyrrole]: Non-steroidal androgen-receptor

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antagonist produced by *A. Pseudomonas*" J. Antibiotics 46(9):1327-1333 (1993) describe the nonsteroidal androgen receptor antagonist labeled WB2838:

WB2838



N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide is described by T. Wong "Synthesis and Some Related Studies of Alkyl 2,3-bis(alkylidene)cyclopentane carboxylates", Ph.D. thesis, University of British Columbia, December, 1993. No use for this compound is described in the thesis.

Steroid hormones are involved in numerous aspects of cell growth and differentiation. In an effort to influence these processes chemists have developed analogs usually based on the four ring steroid nucleus. Nonsteroidal compounds interacting with steroid receptors are more rare and exhibit different pharmacological properties from their steroidal counterparts. We describe the use of androgen receptor binding assays and androgen-dependent DDT1 cells to identify and characterize novel antiandrogens, of particular interest are nonsteroidal antiandrogens.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the results of two independent runs of the experiment described in Example 1. This graph shows the androgen dependent growth of DDT1 cells. Cells were plated in a 96 well plate at 2,000 cells/well in DFITS+0.1% fetal calf serum. After 24 hours, the cells were washed with the same medium except without added serum and replaced with fresh serum free DFITS (day 0) with ethanol mock control (open squares), with 10 nM testosterone (open circles), with 10 μ M N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide alone (filled squares), or 10 μ M N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide in the presence of 10 nM testosterone (filled circles). Cell growth data were normalized to the greatest number of cells and error bars represent \pm one standard error of the mean (sem).

FIG. 2 is a graph showing the effect of N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide on the activation of the androgen receptor in CV1 cells, as described in Example 4. The filled triangles represent N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide alone, the filled squares represent N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide plus 10 nM dihydrotestosterone (DHT).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for determining the antiandrogenic action of a compound comprising:

- plating DDT1 cells (ATCC CRL-12051) in medium free from androgens;
- adding a solution containing the compound to the plated DDT1 cells;
- measuring the growth of the DDT1 cells in the presence of the compound over a measured period of time.

In one embodiment of the present invention, the method for determining the antiandrogenic action of a compound comprises:

- (a) plating DDT1 cells (ATCC CRL-12051) in medium free from androgens;
- (b) adding a solution containing the compound to the plated DDT1 cells;
- (c) measuring the growth of the DDT1 cells in the presence of the compound over a measured period of time and calculating a rate of growth of the DDT1 cells.

In one aspect of the present invention, the DDT1 cells are grown in synthetic serum-free media. The DDT1 cell line, also referred to as the DDT1 MF-2 cell line, was an existing deposit with the American Type Culture Collection ATCC No. CCRL-1701 which has been re-deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. United States of America under the conditions of the Budapest Treaty as ATCC CRL-12051 on Feb. 21, 1996. The DDT1 smooth muscle cell line was cloned from cells derived from a leiomyosarcoma of the ductus deferens of a Syrian hamster (*Mesocricetus auratus*). It has maintained androgen and glucocorticoid receptors and alpha 1 and beta 2 adrenergic receptors. The beta 2 adrenergic receptor is functionally coupled to adenylate cyclase. Glucocorticoids regulate the expression of the c-sis proto-oncogene (inhibit). Growth is stimulated by androgens and inhibited by glucocorticoids.

Preferably, the method of the present invention is carried out by comparing the rate of growth of DDT1 cells in the presence of the compound to the rate of growth of DDT1 cells in the absence of the compound. In one embodiment of the present invention, a known volume of the solution containing the compound is added to the plated DDT1 cells. In a further illustration of the present invention, the solution of the compound is of a known concentration. When a known volume of a known concentration of the compound is added and the resulting rate of growth of the DDT1 cells is compared to the rate of growth when a "blank" solution is added (a volume of the solvent used to dissolve the compound equal to the volume of the solution containing the compound in the comparative run), the IC_{50} of the compound may be computed. The compound may be dissolved in a suitable solvent such as DMSO, ethanol, water or methylethyl ketone (MEK). Preferably, the compound is dissolved in ethanol to form an ethanol solution. In one aspect of the present invention, a known volume of a known concentration of the ethanol solution is added. In a further aspect of the present invention, the rate of growth of the DDT1 cells when a known volume of a known concentration of the compound in an ethanol solution is compared to the rate of growth of DDT1 cells when the same volume of ethanol is added.

In one embodiment of the present invention, the growth of the DDT1 cells is measured over the period of one hour.

In one class of the present invention, the measurement of the growth of the DDT1 cells over the measured period of time is performed by counting the number of cells at the beginning and at the end of the period of time. This can be done by quantitating cells using a hemocytometer.

In another class of the present invention, the measurement of the growth of the DDT1 cells over the measured period of time is performed by incubating the DDT1 cells with labeled thymidine and measuring the incorporation of the label as a measure of DNA synthesis. Preferably, the labeled thymidine is 3H -thymidine, and the incorporation of the tritium label is measured by incubating cells for a period of time, preferably overnight, and washing extensively to

remove free 3H -thymidine. Cells are then removed, preferably using trypsin, resuspended in media and the radioactivity quantitated, preferably by scintillation counting, for example using a Tri-Carb 2500TR Liquid Scintillation analyzer manufactured by Packard.

In still another class of the present invention, the measurement of the growth of DDT1 cells is measured by colorimetric analysis. In particular, the growth may be measured by diluting 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium into the medium and adding phenazine methosulfate, and measuring the absorbance at 490 nm.

In one embodiment of the present invention, the cells are plated in 96 well plates. In a preferred class of this embodiment of the invention, the assay is automated; i.e., the solutions are dispensed using a robotic device.

In one embodiment of the method of the present invention, the tested compound is assayed to determine that the compound inhibits the binding of labeled dihydrotestosterone (DHT) to the androgen receptor (AR). The androgen receptor used in the assay can be obtained from tissue such as prostate or liver, preferably human tissue, or cloned from the known sequence, see Tilley et al., Proc. Nat'l. Acad. Sci. USA, 80:327-331 (1989). Alternatively, the source of the human androgen receptor can be a cell line transfected with the human androgen receptor. Techniques for obtaining these transfected cell lines are well-known in the art and are preferred because of the uniformity of the receptor produced. The androgen receptor is incubated with labeled DHT, particularly 3H -DHT, and the compound to be tested or unlabelled DHT is added to the incubated receptor. The unlabelled DHT may be run in a parallel experiment in order to quantitate the nonspecific binding. The affinity for the androgen receptor is then calculated. One method of calculating this affinity is to add an agent to remove free labeled DHT. This may be done by a charcoal technique by adding dextran-coated charcoal to the sample at 0.5 volume of the original assay volume, vortexing, and centrifuging at 3,000xg for 15 minutes at 4° C. Bound labeled DHT (preferably 3H -DHT) is in the supernatant. In a filter binding assay, the bound androgen receptor-labeled DHT (preferably AR- 3H -DHT) complexes can be precipitated using agents such as ammonium sulfate, and filtered through glass fiber membranes. After extensive washing, bound labeled DHT (preferably 3H -DHT) is measured. When 3H -DHT is employed, the remaining label may be measured by scintillation counting of the glass fiber membranes, for example using a Tri-Carb 2500TR Liquid Scintillation analyzer manufactured by Packard. The compound may be tested for its ability to inhibit binding of DHT to the androgen receptor either before or after determining the antiandrogenic action of the compound according to the method of the present invention.

The present invention presents a convenient assay for monitoring the effect of compounds in a tissue culture system and demonstrates that the result of such an assay is the discovery of a nonsteroidal compound that acts as an antiandrogen.

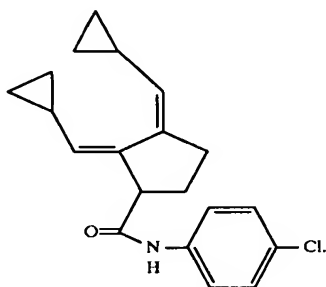
Further, the present invention relates to a method of treating or preventing diseases of the prostate comprising administering 0.001 to 200 mg per day of a compound capable of inhibiting androgen-induced DDT1 cell growth to a male human in need of such treatment.

The instant invention involves a method of treating and/or preventing BPH and prostatic carcinoma which comprises administering to a patient in need of such treatment a therapeutically effective amount of a compound which

inhibits the testosterone induced growth of DDT1 cells. In one embodiment of this method, the compound which inhibits the testosterone-induced growth of DDT1 cells is administered in a dosage amount between 0.001 to 200.0 mg/day. In one class of this embodiment, the compound which inhibits the testosterone-induced growth of DDT1 cells is administered in a dosage amount of from 0.01 to 50.0 mg/day, and in a sub-class of this embodiment, the compound which inhibits the testosterone-induced growth of DDT1 cells is administered in a dosage amount of about 0.1 to 5.0 mg/day. Compounds which inhibit the testosterone-induced growth of DDT1 cells can be determined by employing the assay described in Example 1.

In a second embodiment of this invention, the methods of treating and preventing benign prostatic hyperplasia and prostatic carcinoma comprise administration to a patient in need of such treatment of a compound which inhibits the testosterone-induced growth of DDT1 cells. Preferably, this compound is a non-steroidal compound.

Preferred compounds that may be employed in the present invention include N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide of structural formula (I):



and pharmaceutically acceptable salts, esters and prodrugs thereof.

The nonsteroidal compound N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide which antagonizes the action of testosterone on DDT1 cells but exhibits little or no effect on cell growth by itself. This compound also blocks the binding of ^3H -DHT to the human androgen receptor expressed in stable transfectants of CHO cells at doses comparable to those that antagonize testosterone in DDT1 cells. This compound may thus represent a novel class of nonsteroidal antiandrogens which show promise in the treatment of prostate cancer.

The present invention has the objective of providing methods of treating and preventing diseases of the prostate including BPH and prostatic carcinoma by systemic, oral, parenteral or topical administration of a compound which inhibits the testosterone-induced growth of DDT1 cells in a dosage amount between 0.001 to 200.0 mg/day, and more particularly, from about 0.01 to 50.0 mg/day, and most particularly 0.1 to 5.0 mg/day. The term "treating BPH" is intended to include alleviating the obstructive symptoms of BPH, and slowing and/or reversing the growth of the prostate. The term "preventing BPH" is intended to include preventing development of obstructive symptoms, and preventing the enlargement of the prostate. The term "treating prostatic carcinoma" is intended to include slowing and/or stopping the growth of prostatic carcinoma. The term "preventing prostatic carcinoma" is intended to include preventing the development of prostatic carcinoma in patients likely to develop prostatic carcinoma. Also, a compound which inhibits the testosterone-induced growth of DDT1 cells may be co-administered with a 5α -reductase 2 inhibitor, such as

finasteride or epristeride; a 5α -reductase 1 inhibitor such as 4,7 β -dimethyl-4-aza- 5α -cholestan-3-one, 3-oxo-4-aza-4,7 β -dimethyl-16 β -(4-chlorophenoxy)- 5α -androstan-3-one, and 3-oxo-4-aza-4,7 β -dimethyl-16 β -(phenoxy)- 5α -androstan-3-one as disclosed in WO 93/23420 and WO 95/11254; dual inhibitors of 5α -reductase 1 and 5α -reductase 2 such as 3-oxo-4-aza-17 β -(2,5-trifluoromethylphenylcarbonyl)- 5α -androstan-3-one as disclosed in WO 95/07927; nonsteroidal antiandrogens such as flutamide and Casodex, and alpha-1 blockers such as prazosin, terazosin, doxazosin, tamsulosin, and alfuzosin. The term "co-administered" includes concurrent administration as well as separate provision of the identified medicaments.

The present invention also has the objective of providing suitable systemic, oral, parenteral and topical pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing as an active ingredient a compound which inhibits the testosterone-induced growth of DDT1 cells can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for systemic administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. For oral administration, for example, the compositions can be provided in the form of tablets containing 0.01, 0.05, 0.1, 0.2, 1.0, 2.0, 5.0, 10.0, 50.0 and 100.0 milligrams of the active ingredient for the adjustment of the dosage to the patient to be treated.

The compound which inhibits the testosterone-induced growth of DDT1 cells may be administered in a pharmaceutical composition comprising the active compound in combination with a pharmaceutically acceptable carrier adapted for topical administration. Topical pharmaceutical compositions may be, e.g., in the form of a solution, cream, ointment, gel, lotion, shampoo or aerosol formulation adapted for application to the skin. Topical pharmaceutical compositions useful in the method of treatment of the present invention may include about 0.001% to 0.1% of the active compound in admixture with a pharmaceutically acceptable carrier.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. The compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter, arrest or reverse the progress of the condition. Optimal precision in achieving concentration of

drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of the drug.

In the methods of the present invention, the compound which inhibits the testosterone-induced growth of DDT1 cells herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Capsules containing the product of this invention can be prepared by mixing an active compound of the present invention with lactose and magnesium stearate, calcium stearate, starch, talc, or other carriers, and placing the mixture in a gelatin capsule. Tablets may be prepared by mixing the active ingredient with conventional tableting ingredients such as calcium phosphate, lactose, corn starch or magnesium stearate. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

The liquid forms may be administered in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methylcellulose and the like. Other dispersing agents which may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations which generally contain suitable preservatives are employed when intravenous administration is desired.

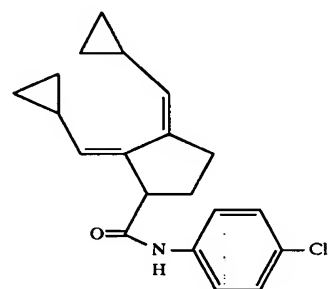
Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations. See, e.g., EP 0 285 382.

The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamidephenol,

polyhydroxyethylaspartamidephenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

Thus, the present invention also provides for a pharmaceutical composition for the treatment of diseases of the prostate comprising a compound capable of inhibiting androgen induced DDT1 cell growth and a pharmaceutically acceptable carrier. In one class of this embodiment of the invention, the compound capable of inhibiting androgen-induced DDT1 cell growth is N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide of structural formula (I):



(I)

The present invention also provides for the use of a compound which inhibits the testosterone-induced growth of DDT1 cells in the preparation of a medicament useful in the treatment of diseases of the prostate.

The following examples are not intended to be limitations on the scope of the instant invention in any way, and they should not be so construed. Furthermore, the compounds described in the following examples are not to be construed as forming the only genus that is considered as the invention, and any combination of the compounds or their moieties may itself form a genus. Those skilled in the art will readily understand that known variations of the conditions and processes of the following preparative procedures can be used to prepare these compounds.

All temperatures given in the following examples are in degrees Celsius.

EXAMPLE 1

DDT1 Assay

DDT1 cells (ATCC Nos. CRL-1701 and CRL-12051) were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL) (DMEM)+2% bovine calf serum (Hyclone®, BCS defined, iron supplemented) and 10 nM testosterone. Media was changed every 48 hours. Cells were plated at ~2,000/well in 96-well plates or ~6,000 cells/cm² in DMEM: F12 (GIBCO BRL) nutrient mixture (1:)+ITS™ (insulin, transferrin, selenious acid) (Collaborative Biomedical Products/Becton Dickinson Labware) (final concentrations 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenious acid) (DFITS media)+0.1% bovine calf serum (Hyclone®, BCS defined, iron supplemented).

After 24 hours, the cells were washed with the same media except without added serum and replaced with fresh serum-free DFITS medium (day 0).

In a typical experiment, cells were plated in sets of 12 in the presence of either a mock ethanol control (0.1–0.2% total volume) or N-(4-chlorophenyl)-(Z,Z)-2,3-bis

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(cyclopropylmethylene) cyclopentane carboxamide dissolved in 100% ethanol as a 1000X concentrated stock solution. Cell growth was then measured using a CellTiter96™ Non-Radioactive Cell Proliferation Assay (Promega Corporation) according to the manufacturer's protocol using a Bio Kinetics EL 340 microplate reader (BioTek Instruments, Inc.). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyl methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was diluted into media at a final concentration of 333 $\mu\text{g/mL}$ and penazine methosulfate (PMS) was used at a final concentration of 25 μM with a total volume per well of 100 μL . Plates were then incubated for one hour at 37° C. in a humidified 5% CO₂ atmosphere. A linear response between cell number and absorbance at 490 nm was found up to 20,000 cells/well. The proliferation of DDT1 cells in synthetic serum-free medium is very slow doubling time >5 days in the absence of added testosterone, but is robust in the presence of 10 nM testosterone as shown in FIG. 1. The addition of either N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide or an ethanol mock control showed little or no effect on cell proliferation. In contrast, the addition of N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide at 10 μM along with 10 nM testosterone reduced cell proliferation to levels close to that of the ethanol mock control. The classical nonsteroidal antiandrogen flutamide at 1 μM had the same effect, within experimental error, of blocking testosterone in this system. Thus N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide inhibits testosterone-induced DDT1 cell growth in a manner similar to that of hydroxyflutamide.

EXAMPLE 2

Binding to the Androgen Receptor

100 μL of cytosol (100,000 \times g supernatant) were incubated overnight at 4° C. in the presence of 1 nM [³H]-DHT with and without unlabelled DHT at 1 μM to assess non-specific binding with and without added compound to measure competition. All samples were prepared in duplicate. Compound stock solutions were typically 100 fold concentrated in 100% ethanol and equal volumes of ethanol were employed as a mock control. Free steroids were removed by addition of dextran-coated charcoal followed by centrifugation at 3,000 \times g for fifteen minutes at 4° C. Receptor-bound [³H]-DHT was then measured by Scintillation counting of the supernatants.

Binding to the human androgen receptor (hAR) was measured essentially as described by Tilley et al., "Characterization and expression of cDNA encoding the human androgen receptor", Proc. Nat'l Acad. Sci. USA 86:327-331 (1989), and Summerfield et al., "Tissue-specific pharmacology of testosterone and 5 α -dihydrotestosterone analogues: characterization of a novel canine androgen-binding protein", Mol. Pharm. 47: 1080-1088 (1995) except that stable transfectants of CHO cells were employed (gift from Prof. Michael McPhaul Southwestern Medical Center, Dallas, Tex.) that typically express 30-80 fmoles receptor/mg soluble protein.

N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide exhibits an IC₅₀ (fifty percent displacement (IC₅₀) of 3H-5 α -DHT (1.0 nM) of 27.5 \pm 0.13 μM (two independent experiments). Hydroxyflutamide has an IC₅₀~100 nM using the same binding assay.

EXAMPLE 3

Preparation of N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide

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To a stirred solution of 4-chloroaniline (66 mg, 0.52 mmol) in dry benzene (2.6 mL, argon atmosphere) was added a solution of trimethylaluminum in toluene (0.26 mL, 2M, 0.52 mmol) and the mixture was stirred at room temperature for 20 minutes. A solution of methyl (Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxylate (80 mg, 0.344 mmol) in dry benzene (2.0 mL) was added and the mixture was refluxed for 4 hours. Hydrochloric acid (2M, 4 mL) was added and the phases were separated. The aqueous phase was extracted three times with diethyl ether. The combined organic extracts were washed (brine), dried (magnesium sulfate) and concentrated. Flash chromatography (20 g silica gel, 4:1 petroleum ether-diethyl ether) of the crude product and removal of traces of solvent (vacuum pump) from the acquired material produced 93.5 mg (83%) of the amide, N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide. Recrystallization of this material from 1:1 dichloromethane-ethanol gave the amide N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide as colorless needle-like crystals (mp 126°-127° C.) that showed IR (KBr): 3255, 1664, 1594, 1494, 1399, 1096, 824 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz): 0.42-0.60 (m, 4H), 0.80-0.95 (m, 4H), 1.77-1.90 (m, 2H), 2.00-2.10 (m, 1H), 2.10-2.22 (m, 1H), 2.35-2.53 (m, 2H), 3.40 (dd, 1H, J=9, 4 Hz), 4.87 (d, 1H, J=10 Hz), 4.96 (d, 1H, J=10 Hz, 7.26 (d, 2H, J=9 Hz), 7.44 (d, 2H, J=9 Hz), 7.56 (br s, 1H, w_{1/2}=8 Hz); in a decoupling experiment, irradiation at δ 1.82 simplified the multiplets at 0.42-0.60 and 0.80-0.95, and simplified the two doublets at 4.87 and 4.96 to two broad singlets (w_{1/2}=5, 3 Hz, respectively); in a series of NOE difference experiments, irradiation at δ 3.40 caused enhancement of the signals at 2.10-2.22 (9%) and 4.96 (10%); irradiation at δ 4.87 caused enhancement of the signals at 1.77-1.90 (4%) and 2.35-2.53 (3%); irradiation at δ 4.96 caused an enhancement of the signal at 3.40 (4%); ¹³C NMR (CDCl₃, 50.3 MHz): δ 6.9, 7.1, 8.5, 8.9, 13.8, 14.1, 26.9, 31.9, 53.3, 120.6, 128.85, 128.9, 130.2, 133.6, 135.6, 136.2, 136.8, 173.3. Exact mass calcd. for C₂₀H₂₂³⁵ClNO: 327.1392; found: 327.1384. Anal. calcd. for C₂₀H₂₂ClNO: C 73.27, H 6.76, N, 4.27; found: C 73.17, H 6.67, N, 4.40.

EXAMPLE 4

Ligand dependent transcription assays were performed as described in Schnmidt et al. "Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids," Mol. Endocrinol. 6:1634-1641 (1992). A luciferase cDNA expressed under the control of the promoter region of the probasin gene that confers androgen receptor dependent transcription was used as a reporter gene, see Rennie et al. "Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene" Mol Endocrinol. 7:23-36 (1993). The promoter region, -426 to +40 of the rat probasin gene was amplified by polymerase chain reaction (PCR) and inserted upstream of the luciferase cDNA of pGL3 Basic plasmid (Promega). The expression vector pSGAR that expressed the normal human AR was used as a source of receptor, see, Chang et al. "Molecular cloning of human and rat complementary DNA encoding androgen receptors," Science 240:324-326 (1988). Transient transfection of CV1 cells was performed by plating cells (1.5 \times 10⁵ per mL) into 12 well dishes in phenol red-free medium supplemented with 10% fetal calf serum treated with activated charcoal to remove endogenous steroids. Cells were then transfected the following day by addition of a calcium phosphate precipitate of plasmid DNA. Cells were then washed with fresh media

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after an overnight incubation and ligands were added. Cell extracts were prepared after forty eight hours and assayed for luciferase enzyme activity using the Luciferase Assay System (Promega). Each transfection was performed in triplicate and the fluorescence of each sample was measured using the AutoChemiluminometer (Berthold).

Thus to test whether N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide acts as an agonist or antagonist to the androgen receptor, the effect of N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide on either the AR dependent transcription from the pPBluc reporter gene or the ability of N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentanecarboxamide to block the androgen receptor transactivation by DHT. The androgen receptor and the probasin luciferase reporter gene were co-transfected into CV1 cells and N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide was added to the cells without or with 10 nM DHT. Treatment using N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide alone did not affect luciferase expression from the pPBluc reporter gene. In contrast, N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide inhibited the transactivation mediated by DHT in a dose dependent manner. Fifty percent inhibition of transactivation occurred at about 2 μ M and maximal inhibition occurred at 100 μ M. The results are graphed in FIG. 2.

TABLE 1

Androgen Receptor (AR) and DHT dependent transcription from the promoter of the probasin gene				
DHT	Light Units \pm std		Fold Stimulation \pm std	
	pPBluc	pPBluc + AR	pPBluc	pPBluc + AR
control	4200 \pm 600	46000 \pm 40000	1.00 \pm 0.13	1.00 \pm 0.08
10 ⁻⁹ M	3000 \pm 300	290000 \pm 40000	0.71 \pm 0.07	6.3 \pm 0.9
10 ⁻⁷ M	3300 \pm 1100	1100000 \pm 70000	0.78 \pm 0.25	24 \pm 1

CV1 cells were co-transfected with the probasin reporter gene (pPBluc) and with either the androgen receptor (pSGAR) or control plasmid (pSV2neo) and treated with vehicle or with DHT at the indicated concentration as described above.

EXAMPLE 5

Oral Composition

As a specific embodiment of an oral composition of a compound of this invention, 5 mg N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide, is formulated with sufficient finely divided lactose to provide a total amount of 580 to 590 mg to fill a size 0 hard gelatin capsule.

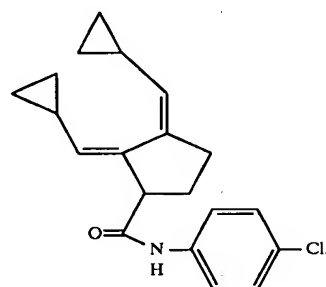
While the invention has been described and illustrated with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various changes, modifications and substitutions can be made therein without departing from the spirit and scope of the invention. For

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example, effective dosages other than the particular dosages as set forth herein above may be applicable as a consequence of variations in the responsiveness of the mammal being treated for any of the indications for the compounds of the invention indicated above. Likewise, the specific pharmacological responses observed may vary according to and depending upon the particular active compound selected or whether there are present pharmaceutical carriers, as well as the type of formulation and mode of administration employed, and such expected variations or differences in the results are contemplated in accordance with the objects and practices of the present invention. It is intended, therefore, that the invention be defined by the scope of the claims which follow and that such claims be interpreted as broadly as is reasonable.

What is claimed is:

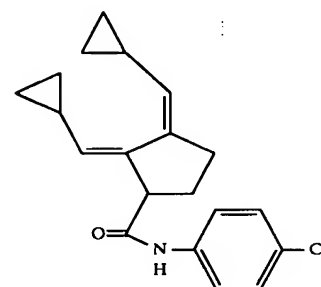
1. A method of treating or preventing diseases of the prostate comprising administering 0.001 to 200 mg per day of a compound capable of inhibiting androgen-induced DDT1 cell growth to a male human in need of such treatment wherein the compound is N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide of structural formula (I):



2. The method of claim 1 wherein the disease of the prostate is prostatic cancer.

3. The method of claim 1 wherein the disease of the prostate is benign prostatic hyperplasia.

4. A pharmaceutical composition for treatment of diseases of the prostate comprising N-(4-chlorophenyl)-(Z,Z)-2,3-bis(cyclopropylmethylene)cyclopentane carboxamide of structural formula (I):



and a pharmaceutically acceptable carrier.

* * * * *

Androgenetic alopecia: pathogenesis and potential for therapy

Justine A. Ellis, Rodney Sinclair and Stephen B. Harrap

Androgenetic alopecia occurs in men and women, and is characterised by the loss of hair from the scalp in a defined pattern. Determining factors appear to be genetic predisposition coupled with the presence of sufficient circulating androgens. The prevalence of this condition is high (up to 50% of white males are affected by 50 years of age) and, although there are no serious direct health consequences, the loss of scalp hair can be distressing. Knowledge of the pathogenesis of androgenetic alopecia has increased markedly in recent years. Pre-programmed follicles on the scalp undergo a transformation from long growth (anagen) and short rest (telogen) cycles, to long rest and short growth cycles. This process is coupled with progressive miniaturisation of the follicle. These changes are androgen dependent, and require the inheritance of several genes. To date, only one of these genes, which encodes the androgen receptor (*AR*), has been identified. Of the many treatments available for androgenetic alopecia, only two (finasteride and minoxidil) have been scientifically shown to be useful in the treatment of hair loss. However, these therapies are variable in their effectiveness. Discovery of the involvement of the *AR* gene, and the identification of other genes contributing to the condition, might lead to the development of new and more effective therapies that target the condition at a more fundamental level.

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Androgenetic alopecia: pathogenesis and potential for therapy

Androgenetic alopecia, which in men is often referred to as male-pattern baldness, is a common form of scalp hair loss that affects most males by old age (Ref. 1). The condition can also affect females; however, this is less well characterised and it remains controversial as to whether the two conditions are the same (Ref. 2). Certainly, the pattern of hair loss is different in women and the prevalence is lower than that in men (Ref. 3). Therefore, in this review article we will focus specifically on male androgenetic alopecia.

The onset of androgenetic alopecia is extremely variable, and appears to be determined by the presence of sufficient circulating androgens and the degree of genetic predisposition (Ref. 4). The condition is not a serious one from a medical perspective; however, the loss of hair is often an unwanted and stressful event for the patient (Refs 5, 6), and therefore might have considerable psychosocial consequences. Effective treatments are being developed and current research efforts, including the search for genes, are yielding much promising new information.

Another common form of alopecia is alopecia areata, which is an inflammatory form of hair loss that is thought to be autoimmune in origin. Both genetic predisposition and environmental influence lead to episodes of patchy terminal hair loss. The loss might completely reverse without leaving any scarring; alternatively, it might progress and result in the total loss of scalp hair (alopecia totalis) or the total loss of body hair (alopecia universalis). Alopecia areata occurs equally in males and females, and does not appear to be androgen dependent (Refs 7, 8).

Prevalence and clinical features of androgenetic alopecia

By 30 years of age, ~30% of white males have androgenetic alopecia; by 50 years of age, 50% are affected (Ref. 1). White males are four times more likely to develop androgenetic alopecia than are males of African origin (Ref. 9).

Hair loss follows a defined pattern, as described by the Hamilton–Norwood scale (Refs 1, 10) (Fig. 1), beginning with bitemporal recession of the frontal hairline. This is followed by diffuse thinning over the vertex (top) of the scalp, eventually leading to complete hair loss in this region. The bald patch progressively enlarges and eventually

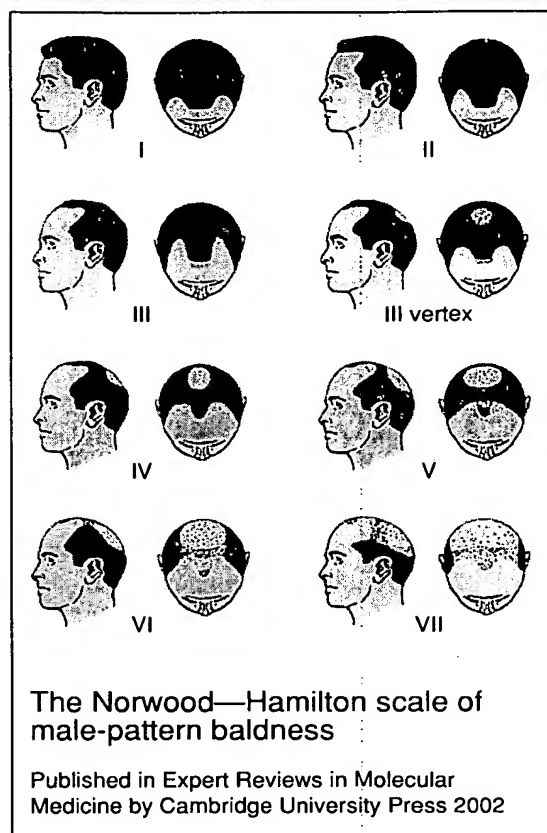


Figure 1. The Norwood–Hamilton scale of male-pattern baldness. The typical pattern of hair loss is divided into seven categories. No hair loss is termed 'type I'. Minor recession of the frontal hairline is termed 'type II'. Type III indicates further frontal loss, and is considered 'cosmetically significant'. The subset of type III, termed 'III vertex', shows significant frontal recession coupled with hair loss from the vertex region of the scalp. Types IV–VI show further frontal and vertex loss, culminating in type VII, in which only the occipital scalp region maintains significant amounts of hair. Reproduced from Norwood, O.T. (1973) *Hair Transplant Surgery* (1st edition), courtesy of Charles C. Thomas, Publisher, Ltd, Springfield, Illinois, USA (fig001jem).

joins the receding frontal hairline. Ultimately, marginal parietal and occipital hair remains, and this might also continue to thin and be lost.

Although this pattern is typical of most cases of androgenetic alopecia, the rate of hair loss in the various scalp regions can produce visual

variation. For instance, rapid vertex loss coupled with slower frontal regression can appear different from faster frontal, and slower vertex, loss. Less commonly, the frontal hairline can be preserved (Ref. 11).

Associations with other conditions

There is some evidence to support the hypothesis that male-pattern hair loss imparts an increased risk of cardiovascular disease (CVD). It would appear that men with androgenetic alopecia are more likely to develop CVD (Ref. 12); however, there is no evidence of an increase in conventional risk factors such as blood pressure and cholesterol concentrations (Ref. 13). Recent studies have also demonstrated an increased risk of benign prostatic hyperplasia (Ref. 14) and prostate cancer (Ref. 15) in those with male-pattern baldness; however, the molecular basis for this link has yet to be evaluated.

Pathogenesis of androgenetic alopecia

Androgenetic alopecia is the result of step-wise miniaturisation of the hair follicle and alteration of the hair-cycle dynamics (Ref. 16). The three phases of the normal hair cycle (Ref. 17) are shown in Figure 2. Pre-programmed follicles on the scalp progress through long growth (anagen) cycles and short rest (telogen) cycles. With each passage through the hair cycle, the duration of the anagen phase decreases whereas the telogen phase elongates. Because the duration of the anagen phase is the main determinant of hair length, the maximum length of the new anagen hair is shorter than that of its predecessor. Eventually, the anagen phase is so short that the emerging hair does not reach the skin surface and the only testimony to the presence of a functioning follicle is a pore. In addition, the latency period between telogen hair shedding and anagen regrowth becomes longer, leading to a reduction

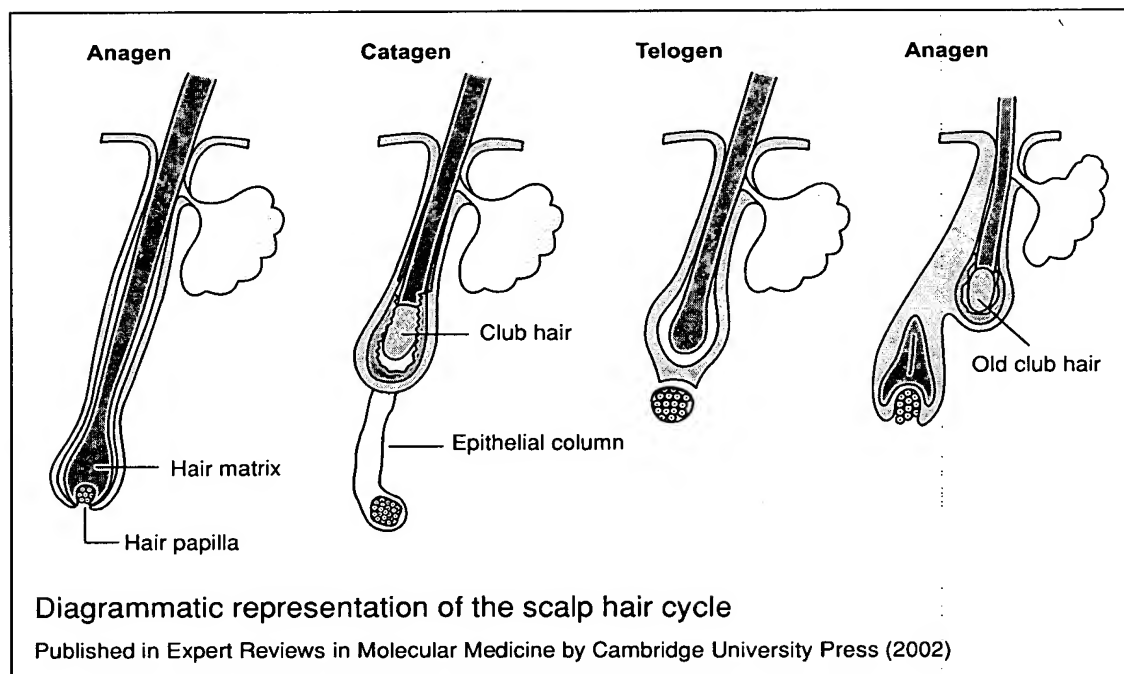


Figure 2. Diagrammatic representation of the scalp hair cycle. (a) During the normal hair cycle, the active growth phase (anagen) can last from 2 years up to 6 years. This is followed by a short transition phase (catagen), which lasts 1–2 weeks, and then by a resting phase (telogen), lasting 5–6 weeks. The hair is then shed, the anagen phase begins again, and a new hair is grown. In the altered hair cycle of the balding scalp (not shown), the phases of the cycle remain unchanged. However, the anagen growth phase becomes shorter and the telogen resting phase becomes longer with each passage through the hair cycle, resulting in diminishing hair length. Reproduced, with permission of the BMJ Publishing Group, from Sinclair, R. (1998) Male pattern androgenetic alopecia, *BMJ* 317, 865–869 (fig002jem).

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in the number of hairs present on the scalp (Ref. 16). The follicular miniaturisation that accompanies these hair-cycle changes is global, affecting the papilla, the matrix and ultimately the hair shaft. The dermal papilla is fundamental to the maintenance of hair growth (Ref. 18) and is probably the target for androgen-mediated changes in the hair cycle and miniaturisation of the follicle (Refs 19, 20) (see below).

Involvement of androgen and the androgen receptor

Two predominant, naturally occurring androgens are the sex steroids testosterone and 5 α -dihydrotestosterone (DHT). Testosterone is converted to DHT by the enzyme 5 α -reductase (Fig. 3a), which exists as two isozymes: type I and type II (Ref. 21). Although the tissue distribution of the isozymes does vary, both are found in scalp follicles (Ref. 22). Androgens mediate their activities by binding to the human androgen receptor, a member of the steroid-thyroid hormone nuclear receptor superfamily. The structure of the androgen receptor includes a ligand-binding domain and a DNA-binding domain. Both testosterone and DHT can bind to the ligand domain, which activates the DNA-binding domain. The receptor–ligand complex then acts as a transcription factor, regulating the expression of androgen-sensitive genes (Fig. 3a) (Ref. 23). The androgen receptor is required for male development and, throughout adult life, for the normal functioning of organs such as the reproductive system, testes, muscles, liver, skin, nervous system and immune system (Refs 24, 25). The androgen receptor plays a role in several diseases and hereditary traits including prostate cancer, androgen insensitivity syndrome (Ref. 26) and spinal and bulbar muscular atrophy (Kennedy disease) (Ref. 27).

The involvement of androgens in androgenetic alopecia has been established for some time, and is well accepted. Eunuchs, who lack androgens, do not bald (Ref. 4). Individuals who lack a functional androgen receptor are androgen insensitive and develop as females; again, these individuals do not bald (Ref. 28). Similarly, no baldness is seen in pseudohermaphrodites, who lack 5 α -reductase, the enzyme that converts testosterone to the potent androgen DHT (Ref. 29). The concentration of DHT has been shown to be higher in the balding scalp than in the non-balding scalp (Fig. 3b) (Ref. 30). In addition, increased

concentrations of both 5 α -reductase and the androgen receptor have been detected in the balding scalp (Ref. 31), suggesting that such changes contribute to hair loss. The exact mechanism(s) through which androgens act to cause baldness remain unclear; however, given that the complex formed between the androgen receptor and androgen acts as a transcription factor, it is likely that genes controlling follicle cycling are regulated by androgen. The expression of such genes will therefore be dependent on the concentrations of androgen and androgen receptor in the follicle (Fig. 3b).

Genetic models of androgenetic alopecia

Androgenetic alopecia – an autosomal dominant disorder?

The nature of the inheritance of genetic predisposition to androgenetic alopecia is unresolved. Although it is a popular belief that baldness is inherited from the maternal grandfather, the mode of inheritance is usually cited in the scientific literature as autosomal dominant, suggesting that the inheritance of only one autosomal gene conveys full genetic predisposition. However, there appears to be only one published comprehensive familial analysis of androgenetic alopecia, conducted by Dorothy Osborn in 1916 (Ref. 32). In this study of 22 families, it was concluded that a single autosomal gene, termed 'B', could account for genetic predisposition to baldness, acting in an autosomal dominant manner in men, and in an autosomal recessive manner in women. In other words, men are predisposed to baldness if they inherit either 'BB' or 'Bb'; however, women are predisposed only if they inherit 'BB'. The inheritance of androgenetic alopecia remains listed as autosomal dominant in such respected references as Victor McKusick's Online Mendelian Inheritance in Man (OMIM: <http://www.ncbi.nlm.nih.gov/Omim>; entry number 109200). However, the first real test of Osborn's hypothesis appeared in 1984, when Kuster and Happle re-assessed the inheritance of androgenetic alopecia (Ref. 33). They presented a strong argument for a more complex polygenic mode of inheritance of androgenetic alopecia, as described below.

The polygenic hypothesis

A trait determined by a single gene would be more likely to display two or more distinct phenotypes,

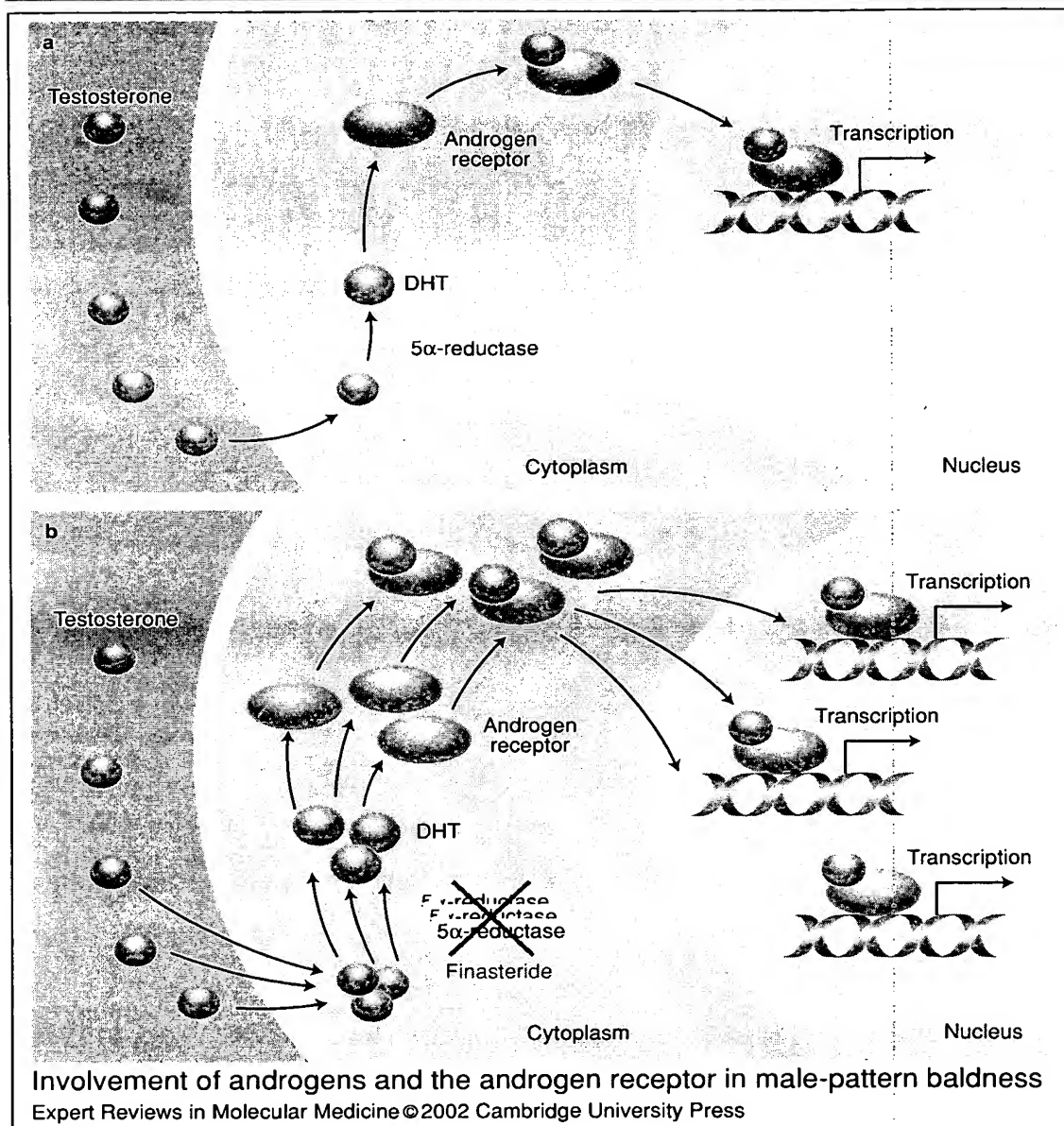


Figure 3. Involvement of androgens and the androgen receptor in male-pattern baldness. (a) In the non-balding scalp, testosterone enters the cell and is reduced to 5α-dihydrotestosterone (DHT) by 5α-reductase. DHT binds to the androgen receptor, and the complex moves into the nucleus, where transcription control of androgen-dependent genes occurs. (b) In the balding scalp, the concentration of 5α-reductase is increased, resulting in the increased production of DHT. Because the concentration of the androgen receptor also appears to be increased, more complexes are formed between androgen receptors and DHT, augmenting the regulation of androgen-dependent genes in the nucleus. The androgen-responsive genes that are involved in male-pattern baldness are yet to be identified. Finasteride, a type-II 5α-reductase inhibitor, reduces the production of DHT by blocking the action of the type II enzyme, thereby slowing the action of the androgen receptor. The site of action of minoxidil is yet to be determined. Future therapies might involve the prevention of the binding of DHT to the androgen receptor (fig003jem).

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rather than a continuous distribution (Ref. 34). In Kuster and Happle's analysis, the distribution of androgenetic alopecia followed a normal distribution, representing the full range of phenotypes, from no evidence of hair loss, to fully developed baldness. This distribution is more consistent with a polygenic trait. In addition, Kuster and Happle pointed out that hereditary traits determined by a single gene rarely occur at a frequency greater than 1 in 1000. Although the exact frequency of androgenetic alopecia is difficult to ascertain, it has been estimated to be between 40% and 60% in men, further supporting a more pervasive mode of inheritance (Ref. 33).

The characteristics of a polygenic trait can be illustrated using a simplified four-gene model (Ref. 35). Consider a scenario whereby predisposition to androgenetic alopecia is determined by four distinct genes, each contributing 25%. Inheritance of none of these genes obviates genetic predisposition to androgenetic alopecia. Inheritance of one gene predisposes to some hair loss later in life. Inheritance of two or three of these genes predisposes to hair loss during middle age, whereas inheritance of all four genes predisposes to hair loss at a young age. In reality, androgenetic alopecia might depend on more or fewer than four genes, each of which might contribute variably to predisposition.

Father-to-son transmission

A recent study of the inheritance of baldness examined fathers and sons who had participated in the Victorian Family Heart Study (VFHS) – a study of 3000 healthy Caucasian individuals belonging to 828 family groups living in the state of Victoria, Australia (Ref. 36). The VFHS was designed to examine risk factors associated with CVD that might include androgenetic alopecia. Males were surveyed for degree of baldness, and concordance was examined between 54 balding adult sons and their fathers. Under a model of autosomal dominant inheritance, sons might be expected to inherit baldness equally from their mothers and fathers. However, 81.5% of balding sons were found to have fathers with cosmetically significant baldness (type 3 or greater on the Hamilton–Norwood scale), considerably exceeding the autosomal dominant expectation (Ref. 36). These findings are consistent with a polygenic mode of inheritance, which includes a paternally inherited

gene, and this polygenic model remains the most likely basis of androgenetic alopecia.

Genes involved in androgenetic alopecia

The identification of genes that are involved in androgenetic alopecia has been hindered by difficulties geneticists have faced when using classical genetic analysis techniques to study the condition. In general, these techniques compare the DNA sequences of unaffected and affected individuals, either from large family groups in which the presence or absence of baldness is known for all family members, or from large groups of unrelated affected and unaffected individuals. In both cases, it is vitally important that the presence or absence of baldness is correctly diagnosed. This is difficult given the variable age of onset of the condition. For example, is a 30-year-old man with a full head of hair guaranteed not to carry genetic predisposition?

In recent studies, the sequences of several candidate genes were compared between groups of individuals who were considered to be most and least genetically predisposed to androgenetic alopecia (Refs 36, 37, 38). These are, respectively, young males who have a significant degree of baldness and older males who have no indication of hair loss. Candidate genes for androgenetic alopecia were chosen because of their relevance to the hypothesis that androgens are involved in this form of hair loss.

Candidate genes

The 5 α -reductase genes

As mentioned earlier, the concentrations of both DHT and 5 α -reductase are increased in the balding scalp (Refs 33, 31), whereas the concentration of testosterone remains the same (Ref. 39). The involvement of DHT, rather than testosterone, in the hair-loss process implicates the genes that encode the 5 α -reductase enzymes, *SRD5A1* and *SRD5A2*, in male-pattern baldness because 5 α -reductase converts testosterone to DHT. However, analyses of these genes using case-control association and familial linkage studies have shown that it is unlikely that they contribute to androgenetic alopecia (Refs 36, 40).

The aromatase gene

The enzyme aromatase converts androgens, such as testosterone, to oestrogens, and appears to be present at decreased concentrations in the balding

scalp (Ref. 31). The autosomal gene encoding aromatase, *CYP19*, was compared between cases and controls but no differences were detected (Ref. 37), suggesting that it is unlikely that the aromatase gene is involved in determining predisposition to androgenetic alopecia.

The Y chromosome

The observed father-to-son transmission of androgenetic alopecia raises the hypothesis that a gene on the Y chromosome might contribute to the condition. In addition, the Y chromosome determines sex and the concentrations of sex steroids such as testosterone and DHT (Ref. 41). However, examination of the non-recombining region of the Y chromosome has demonstrated that it is unlikely that causative mutations occur in any genes contained in this region (Ref. 37). Although most of the Y chromosome does not recombine, it remains possible that mutations that contribute to predisposition to androgenetic alopecia occur in genes that are contained on the ends of the Y chromosome, in the so-called pseudoautosomal regions, which recombine with the X chromosome. Further study will be required to determine if this is the case.

Discovery of the first gene associated with androgenetic alopecia: the AR gene

Because an increased concentration of androgen receptor is associated with the balding scalp (Ref. 31), differences in the DNA sequence of the gene encoding the androgen receptor, or in the AR regulatory sequences, might lead to differences in the concentration or activity of the receptor. Such differences might increase sensitivity to DHT in balding individuals, leading to hair loss at an earlier age.

On comparing the AR gene sequence found in case and control individuals, a significant difference was found between the two groups in the frequency of a single base change in the coding region (exon 1) of the gene. This polymorphism does not alter the amino acid sequence of the protein and is therefore unlikely to be functional. However, it is likely that the polymorphism is tightly linked to other functional sequence changes. All but one of the 54 young, bald men studied carried the non-functional variant of the AR gene (Ref. 38). Interestingly, 77% of non-bald men also carried this version of AR, suggesting that AR is necessary but not sufficient for causing baldness (Ref. 38). To date, AR functional

mutations that are associated with androgenetic alopecia have not been located. However, the results of this study nevertheless provide good evidence for the involvement of the AR gene in androgenetic alopecia. The likely functional difference(s) will be those in the sequence of the regulatory regions that cause relatively subtle increases in androgen-receptor production and activity in the cell (Ref. 38).

Further genetic research

If the AR gene is necessary but not sufficient for causing baldness (Ref. 38), it is possible that other genes might be acting in conjunction with AR. For example, genes other than *SRD5A1* and *SRD5A2* that control DHT production remain candidates. Given that the concentration of 5 α -reductase is increased in the balding scalp, candidate genes might include those encoding transcription factors that regulate the production of 5 α -reductase. Such genes have yet to be identified. The many other genes, known and unknown, that are involved in androgen production, regulation and responses might also be involved.

In addition to androgen-related genes, genes that are involved in patterning, signalling and hair-follicle morphogenesis are attracting much attention from researchers. All hair follicles are formed in utero. The spatial relationship of hair follicles is determined by the competing influences of follicle-inducing and follicle-repressing molecules. Interaction between the epidermis and dermis is required. Epidermal signals trigger the formation of the dermal papilla, and subsequent dermal signals direct epidermal downgrowth into the dermis that envelops the dermal papilla and creates the hair follicle. Hair-shaft production from the follicle is regulated by signals from both the dermal papilla and lateral signalling between epithelial cells. These signalling pathways have been recently reviewed (Ref. 42). Important candidate areas include fibroblast growth factor, WNT proteins, β -catenin, LEF1, FOXN1, noggin, bone morphogenic protein 2 and 4, sonic hedgehog and its cognate receptor patched, platelet-derived growth factor A, follistatin and epidermal growth factor.

Therapeutic management of androgenetic alopecia

Without treatment, androgenetic alopecia is a progressive condition. Hairs decrease in number at a rate of ~5% per year (Ref. 43). Apart from

various camouflage and surgical options (which are reviewed in Refs 44, 45), currently only two pharmaceutical treatments exist for the treatment of androgenetic alopecia in males: topical minoxidil and oral finasteride, both approved by the US Food and Drug Administration.

Minoxidil, a vasodilator, was originally used to treat high blood pressure (Ref. 46). However, following observations that patients treated with this drug showed increased hair growth, a topical formulation was developed that arrested the progression of hair loss and promoted the regrowth of a small amount of hair in ~40% of men (4% experienced medium-to-dense regrowth) (Ref. 47). Minoxidil appears to prolong the anagen growth phase by an as yet unknown mechanism, leading to a decrease in hair shedding, but it does not inhibit the biological process. Hence, once treatment is stopped, hair shedding rapidly resumes, with the loss of all minoxidil-stimulated hair growth (Ref. 48).

Finasteride is a synthetic azo-steroid that has been used for the treatment of androgenetic alopecia in males since 1997. It is a potent and highly selective 5 α -reductase type-2 inhibitor (Ref. 49). It binds irreversibly to the 5 α -reductase type-2 enzyme and inhibits the conversion of testosterone to DHT. Thus, although the pharmacological half-life is in the order of 8 h, the biological half-life is substantially greater. The administration of a daily dose of 1 mg reduces concentrations of scalp DHT and serum DHT by 64% and 68%, respectively (Ref. 50). The dose-response curve is non-linear, and therefore higher doses do not lead to significantly increased suppression of DHT or clinical benefit (Ref. 51). After 24 months of continuous use, 66% of patients experienced ~10–25% regrowth of their hair (Ref. 52). Most of the remainder showed no further hair loss, and only a few continued to lose hair. Continued use beyond 2 years does not appear to promote continued hair regrowth; instead the hair density stabilises with the retention of the newly acquired hairs (Ref. 52). If successful, the treatment should be continued indefinitely because the balding process continues once treatment ceases (Ref. 53).

Future potential for therapy

The importance of the elucidation of androgenetic alopecia genes

The discovery of the genes that are involved in predisposition to androgenetic alopecia will

dramatically enhance our knowledge of the mechanisms both at the molecular and cellular level that result in hair loss. For example, identifying the functional sequence change in or around the AR gene will lead to the determination of the exact differences in androgen-receptor proteins between bald and non-bald men. With this knowledge, treatments can be designed that target and reverse these differences, thereby blocking specific hair-loss mechanisms. Current pharmaceutical treatments for androgenetic alopecia do not target specific cellular mechanisms in this way. Rather, they inhibit enzymes involved in the increase of androgens in the balding scalp; thus, they are suppressive rather than curative, with variable rates of success.

The use of androgen-receptor blockers

Although the involvement of the AR gene in androgenetic alopecia is a recent finding, the idea of blocking the action of the androgen receptor in an attempt to prevent the action of excess DHT in the scalp is not new. However, androgen-receptor antagonists that act systemically cannot be used to treat men, owing to the potential risks of gynaecomastia (an excessive development of the male mammary glands), feminisation and impotence. Therefore, methods must be developed that block the action of the androgen receptor only in scalp follicles.

Knowledge of the sequence differences between the AR gene in balding and non-balding men would allow the possibility of gene-therapy techniques that could selectively deliver the non-balding AR gene to hair follicles, preventing hair loss without any systemic effects. This possibility has been advanced by the development of a topical cream containing liposomes to deliver entrapped DNA selectively to hair follicles in mice (Ref. 54). In this study, the *lacZ* reporter gene was successfully targeted to the hair follicles in mice after topical application of the gene entrapped in liposomes, demonstrating the feasibility in the future of the selective and safe targeting to the hair follicles of genes relevant to androgenetic alopecia. In addition, the variability in the age of onset and severity of baldness among individuals indicates that it is likely that various numbers and combinations of predisposing genes will be identified. Assuming this proves to be the case, such treatments could be designed on a case-by-case basis to target precisely those genes involved in each individual.

Conclusions

Despite the recent research efforts aimed at elucidating the mechanisms behind hair loss in androgenetic alopecia, there is some way to go before a thorough understanding of this condition is achieved. Given that androgenetic alopecia is heritable, identification of the genes involved should lead to a thorough understanding of the causes of this condition. Only on the acquisition of such knowledge will it be possible to design treatments that target the causes. Current pharmaceutical treatments are suppressive rather than curative, and success is variable. The recent association of the gene encoding the androgen receptor with androgenetic alopecia might provide an incentive for the development of better treatments; however, completely effective treatments are unlikely to be developed until a thorough understanding of this condition is achieved at the genetic and molecular level.

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Founded in 1996, the Australasian Hair and Wool Research Society is a not-for-profit organisation that aims to disseminate accurate, ethical, information on all diseases of the hair and scalp to the public. The Society's website provides detailed information, including patient brochures, on various hair conditions, and should be of interest to the medical profession as well as to patients.

<http://www alopecia.com.au>

Features associated with this article

Figures

Figure 1. The Norwood–Hamilton scale of male-pattern baldness (fig001jem).

Figure 2. Diagrammatic representation of the scalp hair cycle (fig002jem).

Figure 3. Involvement of androgens and the androgen receptor in male-pattern baldness (fig003jem).

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The Steroid and Thyroid Hormone Receptor Superfamily

RONALD M. EVANS

Analyses of steroid receptors are important for understanding molecular details of transcriptional control, as well as providing insight as to how an individual trans-acting factor contributes to cell identity and function. These studies have led to the identification of a superfamily of regulatory proteins that include receptors for thyroid hormone and the vertebrate morphogen retinoic acid. Although animals employ complex and often distinct ways to control their physiology and development, the discovery of receptor-related molecules in a wide range of species suggests that mechanisms underlying morphogenesis and homeostasis may be more ubiquitous than previously expected.

STEROID AND THYROID HORMONES ACT TO COORDINATE complex events involved in development, differentiation, and physiological response to diverse stimuli. These molecules, through binding to specific intracellular receptors, coordinate the components of behavioral and physiological repertoires by activating the expression of gene networks. Thus, the hormone-receptor complex may function as a key constituent in determining commitment to specific cell lineages, as well as provoking differentiation in already determined cells. The purposes of this review are (i) to establish the historical perspective that associated these molecules with the control of differential patterns of gene expression; (ii) to describe the striking evolution of our understanding of the structure/function relationships between receptors and the implications for regulation of gene activity; and (iii) to present emerging issues on the physiology and the molecular basis of hormone action.

Past

Diseases that we now understand to be associated with defects in steroid and thyroid hormone function were identified relatively early in medical history; it was only since the early part of this century that a foundation for physiological studies was supplied by the isolation and structural analyses of these hormones. It was known from the work of Huxley and others that extracts from thyroids could control the metamorphosis of amphibians, but it was not until 1915 that Kendall was able to crystallize the molecule involved and show that it was composed of two iodinated tyrosine residues (1, 2). Ten years later, both Kendall and Reichstein completed the structural analysis of cortisol purified from the adrenal cortex, which led to the

realization that it was (as are all other steroid hormones) derived from cholesterol (3, 4). While many considered this to be an achievement of modern endocrinology, one is humbled by the fact that Chinese alchemists (5), for medicinal reasons, developed empirical methods between the 10th and 16th centuries to purify steroids to near homogeneity.

From the early 1900s to the present, there has been a tremendous increase in our knowledge of endocrine organs and the diverse physiology that they coordinate (3, 6). Three major classes of steroid hormones have been described on the basis of biological assays: the adrenal steroids (including cortisol and aldosterone), the sex steroids (progesterone, estrogen, and testosterone), and vitamin D₃. These molecules were shown to be profoundly important for correct vertebrate development and physiology and, consequently, each has become a major focus of biological and clinical investigation (7). The adrenal steroids widely influence body homeostasis, controlling glycogen and mineral metabolism as well as mediating the stress response. They have widespread effects on the immune and nervous systems and influence the growth and differentiation of cultured cells. The sex steroids provoke the development and determination of the embryonic reproductive system, masculinize or feminize the brain at birth, control reproduction and reproductive behavior in the adult, and control development of secondary sexual characteristics. Vitamin D is necessary for normal bone development and plays a critical role in calcium metabolism and bone differentiation. Aberrant production of these hormones has been associated with a broad spectrum of clinical disease including cancer.

Both thyroid and steroid hormones can be important in metamorphosis. A thyroidectomized tadpole will not develop to a frog, but addition of thyroxine to the water induces all of the changes for development to a terrestrial adult (1). Similarly, ecdysteroids act as metamorphic hormones in insects (8). It was possible to associate the action of ecdysone directly to induced changes in chromosome structure (8) during ecdysone-induced chromosome puffing, suggesting a link between steroid hormones and activation of gene expression.

How can small, relatively simple molecules elicit such a diversity of complex responses? The first clue was provided by the identification of steroid and thyroid hormone receptors through the use of radioactively labeled ligands in the early 1970s (9). In each case the hormone induced a change in the receptor such that it associated with high-affinity binding sites in chromatin. This, in turn, led to the induction or repression of a limited number of genes (approximately 50 to 100 per cell) (10). Selectivity is achieved, in part, by restricted expression of the different receptors in specific cells and tissues. Because the chromatin structure of each cell type is uniquely organized, different sets of genes may be accessible to the hormone receptor complex.

Attempts were initiated to purify the steroid and thyroid hormone receptors despite the sobering realization that these molecules

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GRE		
MMTV	- 134	TGGTITGGTATCAAAATGTTCTGATCTG - 108
MMTV	- 187	TTTATGGTTACAACTGTTCTTAAAAAC - 161
NGH	+ 87	CCCTTGGGCACAAATGTTCTGAGGGG + 113
MSV	- 171	CATCTGGGGACCACTGTTCTTGGCC - 197
MSV	- 245	TTCACTGTTCATCTGTTCTTGGCCC - 271
hMTIIA	- 268	GCACCTGGGTACACTGTTCTTGGCCCT - 242
TO	- 439	CTCATATGCACAGCGAGTCTAGTCAAG - 413
TO	- 1174	TACTCTCTTTCATGATGTTCTGGCCCA - 1200
TAT	- 2420	TACCGAGCACTGTTTGTCTAGTCTT - 2446
TAT	- 2515	CTCTGCTGTACAGGATGTTCTAGCTAC - 2489
Consensus		
GGTACANNMTGTTCT		
ERE		
xVit	- 338	AAAGTCAGGTCACAGTGACCTGATCA - 315
cVit	- 625	TATTCCTGGTCAGCGTGACCGGAGCT - 607
Oval	- 173	TATTCAGGTAAACAATGTTTCTG - 199
rPri	- 1587	GCATTTTGTCTCACTATGCTCTAGAGT - 1562
Consensus		
GGTCANNMTGACC		
TRE		
rGH	- 185	GTAAGATCAGGACGCTGACCCGACG - 161
CATCANNNNNMTGACC		

Fig. 1. Alignment of nucleic acid sequences in regions corresponding to identified HREs (15-25). Numbering refers to nucleotide position relative to the start of transcription. Arrows indicate dyad axis of symmetry. A consensus sequence is derived from nucleotides conserved with a frequency of 50% or more. Specific references for HREs can be found in Fig. 2. GRE, glucocorticoid response element; MMTV, mouse mammary tumor virus; hGH, human growth hormone; MSV, murine sarcoma virus; hMTIIA, human metallothionein; TO, tyrosine oxidase; TAT, tyrosine aminotransferase; ERE, estrogen response element; xVit, *Xenopus* vitellogenin; cVit, chicken vitellogenin; Oval, chicken ovalbumin; rPri, rat prolactin; TRE, thyroid hormone response element; and rGH, rat growth hormone.

were present in only trace amounts (10^3 to 10^4 per cell) and would thus require enrichments of 10^5 to 10^6 -fold to achieve homogeneity. The development of high-affinity synthetic analogs of the ligands overcame many of the difficulties of receptor isolation and has revolutionized both clinical and biochemical studies (6, 7). By the early 1980s all but the androgen, mineralocorticoid, and thyroid hormone receptors were purified (11, 12). Each receptor undergoes a structural alteration or "transformation" upon hormone binding, which in turn enables DNA binding. Analysis of the purified glucocorticoid receptor revealed that DNA binding and hormone binding properties, although present in a single molecule, could be separated by limited proteolysis, leading to the first suggestion of a domain structure (13, 14).

Purification and biochemical characterization of the glucocorticoid receptor was accompanied by the identification of a variety of glucocorticoid-responsive genes (11, 12, 15). Many of these genes have been isolated and shown to be transcriptionally regulated by glucocorticoids. Gene transfer studies, particularly with the mouse mammary tumor virus (MMTV) promoter and the human metallothionein IIA promoter, identified short cis-acting sequences (about 20 bp in size) that are required for hormonal activation of transcription (16, 17). The attachment of these elements to an otherwise hormone-nonresponsive gene causes that gene to become hormone-responsive (18). These sequences, or hormone response elements (HREs), function in a position- and orientation-independent fashion and thus behave like transcriptional enhancers (19, 20). Unlike other enhancers, their activity is dependent upon the presence or absence of ligand. These studies suggest that transcriptional regulation derives from the binding of hormone-receptor complexes to HRE sites on DNA. This interpretation has been verified by in vitro footprint analyses which reveal that purified glucocorticoid, estrogen, progesterone, and thyroid hormone receptors bind to the

upstream DNA of responsive genes at sites which correspond to the genetically identified HREs (16, 19-25). The apparent dyad symmetry of these elements (Fig. 1) suggests that they interact with receptor dimers.

Present

Comparative anatomy. Analysis of the hormone receptors is essential for understanding both the origins of complex regulatory systems and how they contribute to the maintenance of the organism. The isolation of steroid receptor complementary DNAs (cDNAs) has identified a family of related genes that bind ligands of remarkable diversity. The interaction between steroid receptor genes, the genetic circuits that they control, and their contributions to spatial organization in the embryo and organ physiology in the adult can now be elucidated.

The expression cloning of the human glucocorticoid receptor (hGR) provided the first completed structure of a steroid receptor and revealed a segment with astonishing relatedness to the viral oncogene *erbA* (26-28). This relationship of the hormone receptors to *erbA* was independently confirmed by the cloning of the human estrogen, progesterone, and vitamin D receptors (29-36). Two groups initiated the characterization of the *erbA* proto-oncogene product that led to its startling identification as the thyroid hormone receptor (37, 38). This represented a critical advance, for it suggested a unifying hypothesis for receptor structure and hormone action.

Although steroid and thyroid hormones are neither structurally nor biosynthetically related, the existence of a common structure for their receptors supports the proposal that there is a large superfamily of genes whose products are ligand-responsive transcription factors. The presence of a highly conserved DNA sequence element initiated searches for such cryptic receptor genes. By means of low stringency hybridization techniques at least five new gene products have been identified. Two of these, referred to as estrogen receptor-related genes 1 and 2 (ERR1 and ERR2), are more related to the steroid than to the thyroid hormone receptors but do not bind any of the major classes of known steroid hormones (39). The remaining are closer to the thyroid hormone receptor. Indeed, one of them is a second thyroid hormone receptor (40, 41). Another is the apparent receptor for the vitamin A-related metabolite retinoic acid (42, 43). The third is closely related to the receptor for retinoic acid; although its ligand is not known (44, 45) the receptor has been implicated in the etiology of hepatocellular carcinoma, and has been named HAP.

The recent characterization of the E75 locus from *Drosophila* predicts the existence of a protein with overall structural properties similar to the steroid and thyroid hormone receptors (46). Structural comparisons of the E75 gene product with the vertebrate homologs indicate remarkable relatedness to the thyroid hormone and vitamin D receptors. Perhaps this is a receptor for the insect steroid ecdysone or the isoprenoid juvenile hormone.

Schematic results of molecular cloning studies are presented in Fig. 2 in which the molecules have been aligned on the basis of regions of maximum protein homology (47). The numbers indicate the extent of sequence identity to the hGR. The central core sequence is rich in Cys, Lys, and Arg residues and is highly conserved (homologies ranging from 42 to 94%). The homology in the ligand-binding domain is more graded and generally parallels the structural relatedness of the hormones themselves. Although the NH_2 -terminus is not conserved, it may contribute to important functional differences between receptors.

Functional domains. The classic model for steroid/thyroid hormone action proposes that binding of the ligand to the receptor

induces an allosteric change that allows the receptor-hormone complex to bind to its DNA response element in the promoter region of a target gene. It is this binding that leads to modulation of gene expression. The cloning of receptor cDNAs provides the first opportunity to dissect the molecular basis of steroid action.

The identification of functional domains for hormone binding, DNA binding, and transactivation was facilitated by a screening assay that uses cultured cells transfected with two DNA expression vectors (Fig. 3). The trans-vector provides for the efficient production of the receptor in cells that do not normally express the receptor gene. The cis-vector contains a luciferase gene (or any other easily monitored function) coupled to a hormone-responsive promoter. Applications of hormone or an experimental agonist will activate the luciferase gene, causing light to be emitted from cell extracts. The level of light emitted is directly proportional to the effectiveness of the hormone receptor complex in activating gene expression.

In the case of the glucocorticoid receptor, the cis-vector contains the mammary tumor virus (MTV) promoter, which has a well-characterized glucocorticoid response element (GRE). In the co-transfection assay, expression of the cis-vector is induced about 500-fold in a hormone-dependent fashion. By means of this assay it is possible to investigate the effects of *in vitro* mutations on receptor activity.

Sequence comparisons in combination with related functional studies have given rise to an emerging picture for a common structure for the receptor superfamily gene products (Fig. 2). An unexpected and revealing result from the mutational studies is that loss of a portion of the hormone-binding region of the glucocorticoid receptor engenders a constitutively active molecule (48, 49). The results provide the first mechanistic insight into the process of activation: neither the steroid-binding domain nor the steroid hormone itself is needed for DNA binding or transcriptional enhancement. Instead, it seems that the hormone-binding region normally prevents the domains for DNA binding and transcriptional activation from functioning. The addition of hormone apparently relieves this inhibition (50, 51).

Our initial suspicion was that the DNA-binding domain is included within the highly conserved central core of the protein. Three features supported this suggestion: (i) the clustering of basic residues likely to interact with DNA, (ii) the presence of a Cys-rich motif, and (iii) the high homology of this core among receptors (27). To test this assignment, different parts of this region were mutated (48, 49, 52, 53). Mutants continue to bind hormone, indicating that the structure of the protein is intact; however, they do not bind DNA. A direct proof of function was provided by substituting the putative DNA-binding domain of the human

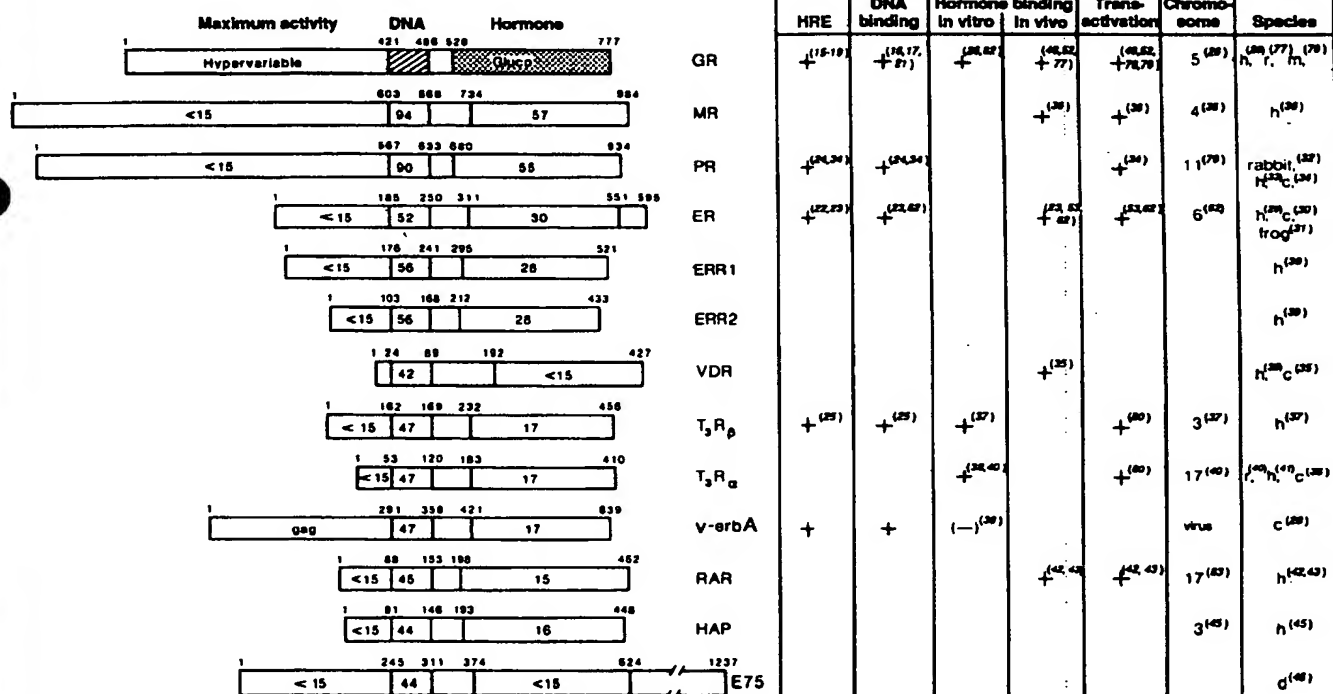


Fig. 2. Schematic amino acid comparison of members of the steroid hormone receptor superfamily. Primary amino acid sequences have been aligned on the basis of regions of maximum amino acid similarity, with the percentage amino acid identity indicated for each region in relation to the hGR (55). Domains shown are a domain at the NH₂-terminal end, required for "Maximum activity"; the 66- to 68-amino acid DNA-binding core ("DNA"); and the 250-amino acid hormone-binding domain ("Hormone"). The amino acid position of each domain boundary is shown. Amino acid numbers for all receptors represent the human forms with the exception of v-erbA and E75 (46). Functional assignments have been determined by characterization of the glucocorticoid and estrogen receptors. Designations are as follows: GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; ER, estrogen receptor; ERR1 or ERR2,

estrogen receptor-related 1 or 2; VDR, vitamin D₃ receptor; and T₃R_β and T₃R_α, thyroid hormone receptors. The (+) or (-) indicates whether a particular property has been demonstrated for the products of cloned receptor cDNA or with purified receptor. HRE, hormone response element. This relates to whether the binding site has been identified structurally and whether its enhancement properties have been demonstrated by gene transfer studies. For PR, DNA-binding properties have been shown only with the native purified receptor. "Hormone binding in vitro" indicates whether this property has been demonstrated by translation in a rabbit reticulocyte lysate system (26). "Hormone binding in vivo" refers to expression of the cloned receptor in transfected cells. "Chromosome" indicates the human chromosome location. Species are as follows: h, human; r, rat; m, mouse; c, chicken; and d, *Drosophila*.

Fig. 3. The cotransfection assay. Cultured cells are transfected with the receptor cDNA in an expression vector (the trans-vector). The function of this transcription factor can be monitored by the activity of a reporter gene (the luciferase gene) linked to an appropriate hormone-responsive promoter. In this case, the promoter is from the MMTV virus carrying a GRE enhancer. The trans-vector encodes the hGR, shown combining with the steroid hormone (triangle).

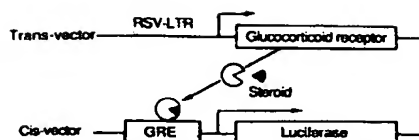
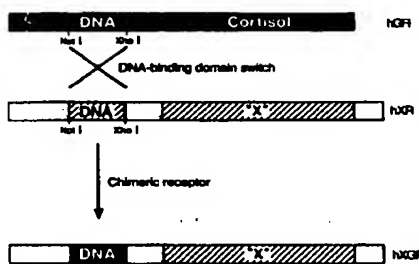


Fig. 4. The finger swap. The modular structure of the steroid receptors allows the exchange of one domain for another to create a functional hybrid. Thus, if the DNA-binding domain of a candidate receptor is substituted with the corresponding region from the glucocorticoid receptor, the resulting chimeric receptor should stimulate the MTV promoter when exposed to the appropriate ligand. This approach was used to functionally identify the retinoic acid receptor (42, 43) and alter the binding specificity of the estrogen receptor (54).



estrogen receptor (hER) with that of the hGR, resulting in a hybrid receptor with the predicted switch in template specificity (54). This suggested a general strategy, referred to as the finger swap, which has been successfully exploited to characterize novel hormone receptors (Fig. 4).

These issues raise the question of whether there are structural aspects of the DNA-binding region that can explain its properties. The most striking feature is the conservation of Cys residues. A comparison of the amino acid sequences in the DNA-binding domain of the hormone receptors (Fig. 5) reveals significant identity and similarity over these evolutionary divergent molecules. Out of 65 residues, 20 are invariant, an additional 7 are conserved in 7/8 of the gene products, and more than half are conserved in 5/8 of the molecules. Nine of the invariant residues are Cys, with one invariant His (Fig. 5). The positioning of the residues is similar to a motif originally observed in the 5S gene transcription factor TFIIIA (55) in which multiple Cys- and His-rich repeating units apparently fold into a "fingered" structure coordinated by a zinc ion (Fig. 5). This finger of amino acids is proposed to interact with a half turn of DNA.

Are such structures important for receptor function? Several results imply they are [see (56) for review]. Site-directed mutagenesis has shown that conserved Cys residues are required for DNA binding (54, 57). Furthermore, recent evidence suggests that the binding of zinc by the receptors is required for DNA binding in vitro (58). Genomic analysis indicates that fingers are encoded by separate exons (36, 59) and an examination of the proposed structure suggests that these fingers are structurally distinct (56). This is most readily seen by the spacing between the cysteines that would be involved in the putative zinc coordination complex. In addition, comparative studies show that the more NH₂-terminal "first finger" is more highly conserved among receptors than the more COOH-terminal "second finger." The first finger is relatively more hydrophilic and has few basic amino acids that might be expected to interact with DNA. The second finger is rich in Lys and Arg residues and is highly basic. Although attention has been

focused on zinc fingers, the residues between the two fingers and the residues immediately after the second finger are also highly conserved. This raises the possibility that these stretches mediate part of the DNA-binding function.

In contrast to the highly conserved DNA-binding domain, the NH₂-terminal extension of the receptors is hypervariable in size and amino acid composition (Fig. 2). Nevertheless, evidence suggests it contributes to function. Deletions in this region of the glucocorticoid receptor reduce activity by 10- to 20-fold (48, 60). Genetic evidence for a functional role for the NH₂-terminus also comes from analysis of the NT¹ (nuclear transfer increased) glucocorticoid receptor mutants (61). NT¹ glucocorticoid receptors appear to contain an altered NH₂-terminus and, although they can bind hormone, they are not biologically functional. Similarly, an estrogen receptor with an NH₂-terminal deletion is able to regulate the vitellogenin promoter in a normal fashion, but is tenfold less active in regulating the expression of the estrogen-responsive promoter p52 (62). Finally, preliminary evidence with the progesterone receptor indicates that the A and B forms, which differ by 128 amino acids at the NH₂-terminus, may have strikingly different capacities to regulate gene expression (63). Such results further support the hypothesis that this domain may modulate receptor function by influencing transactivation, DNA binding, or both.

Subfamilies and superfamilies. The startling discovery of a common structure for the steroid and thyroid hormone receptors and our ability to isolate new receptors by homology suggest that other proteins that contain similar structural features are likely to be hormone- or ligand-responsive transcription factors (LTFs). Apparently it is the analogous action of the hormones that is reflected in the homologous structure of their receptors. An extension of this proposal predicts that other small, hydrophobic molecules may interact with structurally related intracellular receptors. For example, production of cholesterol is regulated by feedback mechanisms that maintain overall levels by monitoring dietary intake and controlling synthesis accordingly (64). Recent evidence demonstrates that at least some of this regulation is transcriptional (65). Since cholesterol is structurally related to steroid hormones, and indeed serves as their biosynthetic precursor, it seems logical to predict both the existence of a cholesterol receptor and its membership in this superfamily. The herbicide TCDD (dioxin) shows close structural relatedness to thyroid hormones and mediates a variety of metabolic effects as a consequence of its action on gene expression. A dioxin receptor has been identified (66), and it now seems likely that this receptor too, will ultimately be part of the LTF superfamily. One of the major issues to arise out of the characterization of this receptor is whether dioxin acts as an agonist or an antagonist for a natural endogenous ligand. Further investigation of this could reveal the existence of a new hormone that may have valuable physiologic and clinical implications.

Preliminary evidence suggests the existence of additional members of the LTF family. For example, the integration of the hepatitis virus in a human liver carcinoma identified a genetic locus (HAP) with striking homology to the DNA-binding fingers of the steroid and thyroid hormone receptors (45). Aberrant expression of HAP could possibly be involved in tumor formation. Indeed, if this locus encodes a new hormone receptor, what might its ligand be? Strong homology to the retinoic acid receptor hints that the product of the HAP locus may bind a related molecule, possibly retinoic acid itself. By extension, the identification of genes for new receptors, by means of low-stringency hybridization techniques, promises to be an exciting area. Already, two novel gene products related to the estrogen receptor have been identified (Fig. 2) (39). Is their expression tissue-specific? Do they bind estrogen? Might they bind other sex steroids and help to identify new hormone response

systems? Such discoveries are likely to have an impact on health and human disease as well as expand our knowledge of basic human physiology.

Remarkably, single ligands may have multiple receptors. Currently, two thyroid hormone receptors have been identified and there may be more (37, 40, 41). What could the advantages be to having different receptors for the same hormone? One possibility is that they are expressed in a tissue-specific fashion. This notion has already been confirmed by the identification of an abundant neuronal form of the thyroid hormone receptor (40). Second, it is possible that they respond differently to thyroid hormone metabolites. Third, since their DNA-binding domains differ slightly, they might activate overlapping, yet partially distinct, genetic networks. Finally, multiple thyroid receptor genes provide multiple promoter enhancers that might be responsive to distinct metabolic or hormonal regulators.

The protein product of *v-erbA* is a derivative of the thyroid hormone receptor that has been proposed to promote leukemogenesis by acting as a thyroxine-independent transcription factor (37, 38). By unknown means, changes in the ligand-binding domain of the protein apparently activate the receptor, perhaps by forcing it into a configuration similar to that achieved by the binding of its physiological ligand. The activation of *erbA* may therefore be an example of how the loss of allosteric control can confer pathogenicity on the product of a proto-oncogene. In vitro studies already indicate that altered glucocorticoid receptors can be biologically active. It thus seems likely that truncations or mutations in other hormone receptors could lead to activated states perturbing homeostatic balance and abetting tumor progression. Although lacking decisive evidence, we can suggest that mutations in the estrogen and androgen receptors may contribute to the conversion of steroid-dependent

breast tumors and prostate tumors to hormone-independent growth (67). As previously mentioned, the integration site of the hepatitis virus in a human tumor may lead to the identification of a new receptor in which a genetic lesion is associated with malignant transformation. A critical step will be the demonstration that mutant receptors contribute to tumorigenesis. It will then be necessary to determine how they exert their effects, whether it simply involves the constitutive activation of hormone-responsive genes or whether it includes an altered substrate specificity so that new genes come under the regulation of mutated receptors. Once a genetic lesion has been identified, this information can be used to contribute to diagnosis and treatment.

Although for many decades it has been understood that sex steroids can influence behavior, the role of other hormones in neurologic function is controversial. Since the 19th-century discovery by Addison of adrenal insufficiency (68), glucocorticoids have been associated with patients' inability to concentrate, drowsiness, restlessness, insomnia, irritability, apprehension, disturbed sleep, and possibly psychotic episodes and manic-depressive disorders. The effects of thyroid hormones on neuronal development and the high level of expression of the thyroid hormone receptor in the adult nervous system lead to the prediction that aberrant hormonal production, variation in receptor expression, or receptor mutations influencing hormone-binding properties could mediate aberrant metabolic effects in the central nervous system (69). Thus, an important future area of investigation is the contribution of receptors to the etiology of psychiatric disorders.

Ontogeny and physiology. Although it is widely believed that differential regulation of gene expression is the critical level at which early development is controlled, this does not provide a conceptual framework for the process by which spatial organization is achieved. Despite excellent evidence for graded positional information in *Drosophila* and nematodes, it is unclear how this relates to morphogenic signals in vertebrates. One long-standing theory is that pattern formation is achieved by the establishment of a gradient of a diffusible substance or morphogen. Work by numerous laboratories over the last several years has indicated that retinoic acid manifests morphogenic properties. Recently, Thaller and Eichele (70) demonstrated that retinoic acid is indeed the substance responsible for establishment of the anterior-posterior axis in the developing chick limb bud. The ability of retinoic acid to induce differentiation in teratocarcinoma cells (71) to parietal endoderm suggests a role for it in the earliest stages of embryonic development.

The discovery of the retinoic acid receptor (42, 43) was made possible from the demonstration that conserved regions in the receptors correspond to discrete functional domains. Thus, by exchanging the DNA-binding domain of the retinoic receptor for the comparable region from the glucocorticoid receptor, a hybrid molecule was generated that activates GRE-responsive promoters (such as the MMTV-LTR) in response to retinoic acid (Fig. 4) (42).

By analogy with steroid receptors, we can propose that the interaction of retinoic acid with its intracellular receptor triggers a cascade of regulatory events that results from the activation of specific sets of genes. Thus, for the first time in a vertebrate system, it should be possible to investigate the mechanism of morphogenesis by identifying a discrete complement of developmental control genes.

With regard to establishment of spatial information, one obvious question is whether there is a gradient of receptor itself. Furthermore, preliminary results reveal the presence of related genes. Might there be receptors for other morphogens and do they also contribute to development?

Mechanisms. What are the molecular interactions between the ligand and the receptor that lead to activation? Once activated, how

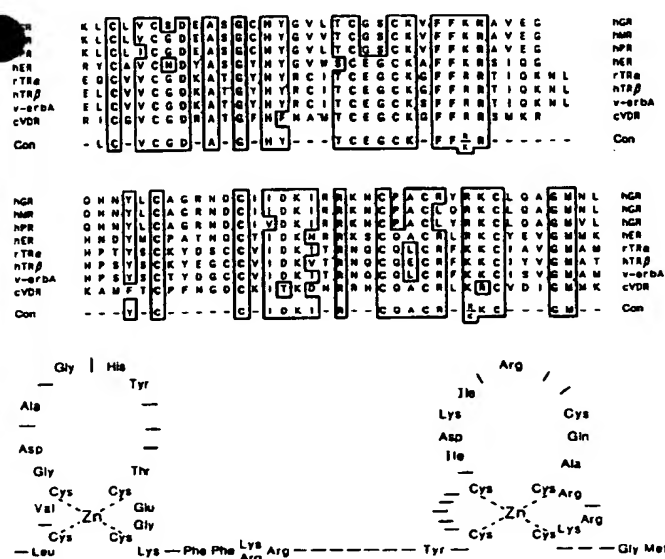


Fig. 5. (Top) Amino acid sequence comparison of DNA-binding domains. A computer program for the concurrent comparison of three or more amino acid sequences was used (47). Amino acid residues matched in at least five of the eight polypeptides are boxed and designated in the consensus (Con) sequence. Hyphens indicate divergent sequences; gaps indicate no comparable amino acids. Absolutely conserved residues are in bold print. **(Bottom)** Hypothetical structure of the DNA-binding domain of the hormone receptors. This domain is configured into two putative zinc-binding fingers with each zinc ion forming a tetrahedral coordination complex with Cys residues. Alternative coordination positions might include the Cys in the second finger and its proximal Cys, shortening the finger and shifting the last conserved Cys into the "trailer" region.

does this molecule find a particular binding site and what is the detailed nature of the DNA-protein interaction? What is the molecular interaction between the receptor and the transcriptional machinery? How do receptors and their potential interaction with other transactivators cause RNA polymerase II to initiate transcription? It must be emphasized that steroid and thyroid hormones can repress gene expression as well as activate it. It is important to determine whether repression and activation are mediated by the same types of DNA sequences and whether other protein factors are involved.

Once a receptor is bound to DNA, how does it activate transcription? Molecular interactions with cognate binding sequences have been analyzed for the transcriptional regulatory proteins lac, λ , and cro (72). Because the DNA-binding domain of the hormone receptors is fundamentally different from that of these molecules (which employ a helix-turn helix motif), it will be necessary to co-crystallize the receptors with cognate DNA-binding sites. These studies, along with site-directed mutagenesis of the receptor, should provide information on how the protein recognizes DNA, but may not reveal the dynamics of transactivation. It will be necessary to determine whether transactivation and DNA binding can be separated as they have been in other regulatory proteins such as GAL4 and GCN4 (73, 74). Assuming these functions are separable, it should be possible to identify receptor variants that bind normally to DNA but fail to transactivate (48, 52, 62). On the basis of this knowledge it will then be necessary to develop techniques to characterize the activation process itself. Does the receptor associate with other transcriptional regulatory proteins? Does this occur before the receptor binds to its HRE? Must the receptor remain bound to the DNA template for the associated gene to remain active or can a transiently bound receptor initiate permanent structural change?

The identification of a transactivation function (τ_1) in the NH_2 -terminus of the glucocorticoid receptor leads to an unexpected conclusion (52). Since the NH_2 -terminus is not conserved among different receptors, they each may achieve this function by distinct means. It has been suggested that the activation domain of yeast GAL4 includes a stretch of acidic amino acids configured in an amphipathic α helix (75). Apparently, overall structural features are critical, rather than the specific sequence. Likewise, the τ_1 region is acidic and so is the activation domain of another yeast regulator, GCN4 (74). It remains to be seen if acidic domains embody the activation function of all the steroid receptors. If so, it might suggest that diverse groups of regulatory proteins from yeast to man employ a remarkably conserved approach to transcriptional control. If the receptors interact with other proteins through acid domains, it will be necessary to purify and characterize these molecules. Ultimately the role of individual components and the mechanism of transactivation must be confirmed by the development of receptor-dependent in vitro transcription systems.

Conclusion

In the 1920s, T. H. Morgan, who explored a genetic approach to development, asserted that to understand development one must understand the molecular basis of differential gene expression (76). Although animals develop in very diverse ways, the discovery of receptor-related molecules in a wide range of species suggests that molecular mechanisms underlying developmental and physiological homeostasis may be much more universal than was previously suspected. The cloning of the steroid and thyroid hormone receptors marks an important step forward in understanding fundamental mechanisms of gene regulation as well as hormone action. The

paradoxical and reciprocal effects of gene regulation on the cell and that of the cell on the gene embody functional physiology in a profound sense. For this paradox reflects both the irreversible changes of embryonic development as well as the recurrent changes in organ physiology in the adult.

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Global Sea Level and Earth Rotation

W. R. PELTIER

Recent analyses of long time scale secular variations of sea level, based on tide gauge observations, have established that sea level is apparently rising at a globally averaged rate somewhat in excess of 1 millimeter per year. It has been suggested that the nonsteric component of this secular rate might be explicable in terms of ongoing mass loss from the small ice sheets and glaciers of the world. Satellite laser ranging and very long baseline interferome-

try data may be used to deliver strong constraints on this important scenario because of the information that these systems provide on variations of the length of day and of the position of the rotation pole with respect to the earth's surface geography. These data demonstrate that the hypothesis of mass loss is plausible if the Barents Sea was covered by a substantial ice sheet at the last maximum of the current ice age 18,000 years ago.

DURING THE PAST 100 YEARS, GLOBAL SEA LEVEL HAS apparently risen by 10 to 15 cm, roughly 50% of which is attributed to the thermal expansion of the oceans (1-3). As recently discussed by Meier (4), the remainder, although usually attributed to ongoing melting of the Antarctic and Greenland ice sheets, is more satisfactorily understood as a product of the present-day retreat of the small ice sheets and glaciers of the world. The reason for this is that the best available information on the contemporary mass balances of the large polar ice sheets suggests that they are near equilibrium, whereas most of the smaller systems are known to be in active retreat. If Meier's hypothesis is correct, it has important implications with respect to climate change since one might attribute the ongoing melting of the small ice sheets and

glaciers to the climate warming expected on the basis of the increase in atmospheric concentrations of CO₂ and other "greenhouse" gases that has been taking place since the beginning of the industrial revolution as a result of the burning of fossil fuels.

Relative Sea Level and Glacial Isostasy

Although the tide gauge data that have been employed to infer the existence of the above-noted secular rate of sea level rise are often strongly contaminated by geological processes, certain regions are sufficiently immune from such processes that the tide gauge records from them are especially valuable as measures of the eustatic variation. These records are probably more meaningful as measures of this variation than the direct global average of the secular rates that has usually been calculated (3). Passive continental margins

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Topical Cyproterone Acetate Treatment in Women With Acne

A Placebo-Controlled Trial

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Objective: To evaluate the clinical and hormonal response of topically applied cyproterone acetate, oral cyproterone acetate, and placebo lotion in women with acne.

Design: Placebo-controlled, randomized study.

Setting: Patients were recruited from the Institute of Endocrine Cosmetics, Vienna, Austria.

Patients: Forty women with acne.

Interventions: Treatment with oral medication consisting of 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate (n=12), 20 mg of topical cyproterone acetate lotion (n=12), and placebo lotion (n=16) was offered. Patients were assessed monthly for 3 months.

Main Outcome Measures: Clinical grading according to acne severity and lesion counts as well as determinations of serum cyproterone acetate concentrations.

Results: After 3 months of therapy with topical cyproterone acetate, the decrease of mean facial acne grade from 1.57 to 0.67 was significantly better ($P<.05$) compared with placebo (which showed a change from 1.57 to 1.25), but not compared with oral medication (1.56 to 0.75) ($P>.05$). Lesion counts also decreased from 35.9 to 9.1 in the topical cyproterone acetate group compared with oral medication (45.4 to 15.5) ($P>.05$) and placebo (38.2 to 23.1) ($P<.05$). After topical cyproterone acetate treatment, serum cyproterone acetate concentrations were 10 times lower than those found after oral cyproterone acetate intake.

Conclusions: The therapeutic effect of topically applied cyproterone acetate for acne treatment was clearly demonstrated. Topically applied sexual steroids in combination with liposomes are as effective as oral antiandrogen medication in acne treatment, while reducing the risk of adverse effects and avoiding high serum cyproterone acetate concentrations.

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HORMONAL approaches to the treatment of acne have been the subject of much interest ever since the sebaceous gland was demonstrated to be sensitive to androgens. The use of topical antiandrogens has had theoretical appeal since the pilosebaceous unit was found to be an androgen-dependent structure. Unfortunately, however, this theory has not been translated into clinical practice.

17 β -Estradiol and progesterone have long been applied topically in the treatment of acne. The reasons for using topical rather than oral therapy include the avoidance of hepatic metabolism, the reduced risk of systemic adverse effects, the ability to combat acne in both female and male patients, and the lack of a contraceptive effect in women. It is known that cyproterone acetate is effective when given orally. However, trials using topical cyproterone acetate have not proved successful

because of the lack of a suitable vehicle for transdermal treatment.^{1,2} Cyproterone acetate is a potent steroidal antiandrogen with progestational activity. It is used alone or in combination with ethinyl estradiol or estradiol valerate in the treatment of women suffering from disorders associated with androgenization, eg, acne or hirsutism. Cyproterone acetate competes with dihydrotestosterone for the androgen receptor and inhibits translocation of the hormone receptor complex into the cell nucleus.³

This study was undertaken to consider once again a cyproterone acetate formulation delivered to the skin surface, thus approaching the target cell topically and bypassing the enterohepatic circulation. The aim of this open, placebo-controlled study was to compare the effectiveness of topical cyproterone acetate, an oral formulation containing 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate (Dianemite, Schering, Vienna, Austria), and a placebo preparation in women with mod-

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PATIENTS AND METHODS

Forty-five women aged between 26 and 38 years (mean age, 30.3 years) with moderate to severe acne who consulted the endocrinology outpatient department for a hormonal evaluation and treatment of their acne were enrolled in this 3-month trial.

Informed consent was obtained from all the patients, and the study was approved by the local ethics committee. Patients with medical contraindications to the therapy or unwilling to smoke less than 5 cigarettes daily were not included in the study. Patients were required to use barrier contraception during the treatment period. All acne medication had been stopped 6 weeks before the commencement of the study.

During the initial visit, the patients were randomly assigned to 1 of the 3 treatment groups. One group ($n=12$) received an oral contraceptive regimen for 3 months, with administration of a daily dose of 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate for the first 21 days of the menstrual cycle, followed by a 7-day pill-free interval. The first treatment cycle started on the first day of menstrual bleeding. The second group ($n=12$) used a topical cyproterone acetate lotion, with treatment starting on the first day of menstrual bleeding and being taken continuously for 3 months. The topical lotion was self-applied once daily in the evening, to the face only, with a calibrated pipette. The daily amount of cyproterone acetate liposome lotion used was 10 mL, containing 20 mg of cyproterone acetate. Liposome lotion consisted of soybean oil, eilecithin, glycerol, and oleic acid (Leopold Pharma, Graz, Austria).

The third group ($n=16$) used a placebo liposome lotion containing soybean oil, eilecithin, glycerol, and oleic acid without the active substance; it was applied as in the topical cyproterone acetate group.

Acne severity on the face was graded according to the method of Burke and Cunliffe,⁴ with the individual scores added up to yield a total result. Four sites on the face (chin, forehead, and left and right cheeks) were graded. The mean value from this grading was used for further calculations. In addition, numbers of macules, papules, pustules, nodules, and cysts on the face were counted by the same dermatologist (E.M.K.) at each visit. Lesion counts were defined as the total of comedones and inflammatory lesions. Only 3 of the patients had few inflammatory lesions, and their counts were thus added to the noninflammatory lesions. Acne on other parts of the body was not evaluated in this study. Assessments were done during the midpoint of the menstrual cycle, ie, between days 10 and 19, with day 1 being defined as the first day of menstrual bleeding. Before commencement of the trial, all patients underwent 2 baseline examinations performed 1 week apart. Progress assessments were made midway through the first, second, and third cycles of treatment. Forty women completed the trial, and their data were included in the final analysis. Five patients dropped out before the end of treatment.

TOPICAL APPLICATION OF CYPROTERONE ACETATE

The application system was similar to that used for topical 17 β -estradiol and topical 17 α -dihydrotestosterone studies. Pharmacokinetic details for these steroidal substances have been described previously.^{5,6} Because cyproterone acetate is a lipophilic steroid, a similar absorption rate was assumed. The daily transdermal cyproterone acetate dosage was 20 mg, based on a similar formulation combining soybean oil, eilecithin, glycerol, and oleic acid.

Liposomes were used to improve the transdermal penetration of the antiandrogen. Topical application of cyproterone acetate in patients with acne had been attempted before, but the clinical results were discouraging.

SERUM CYPROTERONE ACETATE LEVELS

Serum levels of cyproterone acetate were determined in 5 patients in the topical cyproterone acetate group both before treatment and 3 months after study entry, with blood samples taken exactly 45 minutes after the last topical cyproterone acetate application. Baseline serum cyproterone acetate concentrations were below the detection limit. Serum cyproterone acetate levels were measured by radioimmunoassay, by a dextran-coated charcoal method. Aliquots of serum (0.1 mL) were diluted with physiological saline to a final volume of 0.5 mL. After extraction with 2.5 mL of diethyl ether, the ether phase was separated and fully evaporated, and the residue was reconstituted with 0.8 mL of an assay buffer. This solution was incubated with 0.1 mL of tritiated cyproterone acetate (specific activity, 0.84 GBq/mg; Schering AG, Berlin, Germany) and 0.1 mL of antiserum (batch C003, Schering AG) at 4°C for 16 hours, mixed with 0.2 mL of dextran-coated charcoal, and vortexed. After 15 minutes at 4°C, the mixture was centrifuged. The supernatants were decanted into liquid scintillation vials and mixed with 4.5 mL of scintillation cocktail. The radioactivity was measured on a scintillation counter. To obtain the standard curves, 1 mg of cyproterone acetate was dissolved in methanol and diluted with assay buffer, yielding final concentrations ranging from 39 to 10 000 pg/mL. In addition, a drug-free sample (0 pg/mL) was used. Calibration and samples were analyzed in duplicates. A spline function was used for the evaluation of the data. The interassay and intra-assay coefficients ranged from 5.0% to 13.0%. The mean blank value for blank control samples was found to be 28 pg/mL.

STATISTICAL METHODS

Overall significance of the group effect was evaluated by analysis of variance. Pairwise comparisons between groups were made by Student *t* tests for mean comparison. A *P* value less than .05 was considered to indicate significance. The program used for statistical analysis was SAS/PROC GLM (SAS/STAT software, version 6, SAS Institute Inc, Cary, NC, 1989).

erate to severe acne. Moreover, we evaluated in a preliminary study the serum cyproterone acetate levels after transdermal application and compared them with levels obtained by administration of an oral preparation containing 0.035 mg of ethinyl estradiol and 2 mg of cyproterone acetate.

RESULTS

Of the 45 patients who entered the study, 40 completed the 3 cycles of treatment and were included in the analysis (12 receiving oral cyproterone acetate, 12 receiving topical cyproterone acetate, and 16 receiving

Table 1. Clinical Features of Patients in the Trial

Therapy	No. of Patients Entering Trial (No. Completing)	Mean (Range)									
		Age, y	Weight, kg	Facial Acne Grade				Lesion Count			
				Baseline	1 mo	2 mo	3 mo	Baseline	1 mo	2 mo	3 mo
Oral cyproterone acetate	14 (12)	29.4 (26-37)	58.2 (55.7-61.4)	1.56 (1.55-1.58)	1.22 (1.18-1.24)	1.15 (1.12-1.17)	0.75 (0.20-0.97)	45.4 (30-58)	33.5 (21-42)	24.8 (16-35)	15.5 (11-21)
Topical cyproterone acetate	13 (12)	31.3 (26-38)	63.1 (55.3-77.3)	1.57 (1.56-1.58)	1.16 (1.10-1.18)	1.12 (1.10-1.15)	0.67 (0.59-0.80)	35.9 (28-59)	21.8 (12-40)	17.7 (9-33)	9.1 (3-20)
Placebo	18 (16)	30.3 (26-38)	67.0 (55.1-77.5)	1.57 (1.54-1.59)	1.39 (1.30-1.45)	1.30 (1.28-1.40)	1.25 (1.22-1.28)	38.2 (29-47)	32.4 (25-42)	27.6 (21-35)	23.1 (17-30)

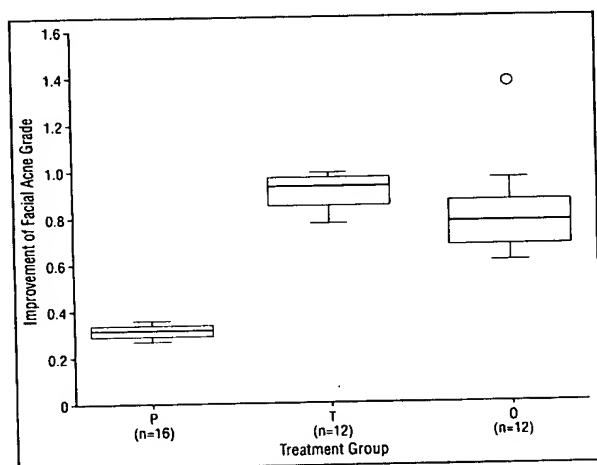


Figure 1. Boxplot of response of the 3 treatment groups in facial acne grades during 3 months of treatment. Mean values (ranges) are as follows: group T, 0.90 (0.77-0.99); group O, 0.81 (0.61-1.37); and group P, 0.32 (0.27-0.36).

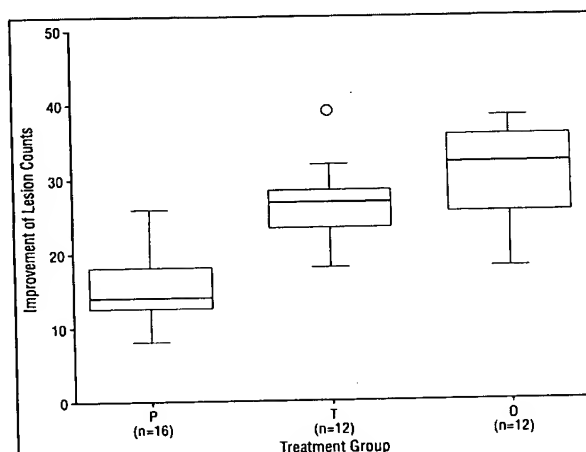


Figure 2. Boxplot of response of the 3 treatment groups in lesion counts during 3 months of treatment. Means (ranges) are as follows: group T, 26.8 (18.0-39.0); group O, 29.9 (18.0-38.0); and group P, 15.1 (8.0-26.0).

placebo). Two patients in the oral cyproterone acetate group dropped out of the study because they had failed to take the tablets regularly. One patient was excluded from the topical cyproterone acetate group because of a moderate local reaction and because she was not willing to use the mechanical contraception. Two patients in the placebo group withdrew from the study for personal reasons. The patients' ages, their initial mean acne gradings, and lesion counts are listed in **Table 1**.

Response to therapy was measured by calculating the difference between baseline measurements and measurements after 3 months of therapy. During the 3-month assessment period, both oral and topical cyproterone acetate were found to be significantly more effective than the placebo preparation in reducing mean facial acne grades and lesion counts ($P < .05$ for both groups). No significant differences in treatment success were observed between the oral and topical therapy ($P > .05$) (**Figure 1** and **Figure 2**).

After 3 months of topical cyproterone acetate application, lesion counts had decreased from a mean of 35.9 (range, 28-59) to 9.1 (3-20) ($P < .05$). The serum levels of cyproterone acetate are shown in **Table 2**. Serum cyproterone acetate concentrations were below the detection limit of 28 pg/mL before the treatment but increased to values of between 119 and

Table 2. Serum Cyproterone Acetate Levels Before and After Topical Cyproterone Acetate Therapy in 5 Patients

Patient No./ Age, y	Serum Cyproterone Acetate, pg/mL	
	Before Treatment	After 3 mo of Treatment
1/34	<28*	226
2/37	<28	281
3/26	<28	286
4/35	<28	311
5/30	<28	119

*Detection limit.

311 pg/mL after topical cyproterone acetate application. No subjective adverse effects or bleeding irregularities were observed in either of the groups during the 3 months of treatment.

COMMENT

Acne is a common disorder in young females and males, and the therapeutic strategies available at the moment are not always sufficient and without shortcomings. Therefore, there is still a need for more effective topical therapies, particularly those that are free of harmful ad-

verse effects and easy to handle. There are numerous well-established hormonal and nonhormonal approaches to the treatment of acne. As acne is known to be an androgen-dependent disorder, oral antiandrogen treatment has been shown to be successful. In the present study, we assessed the efficiency of topical cyproterone acetate in patients afflicted with acne. Although the number of subjects in this study was small, patients were specifically selected from a large group of patients with acne on the basis of having moderate to severe lesion scores, and no other visual evidence of hyperandrogenism. Moreover, none of the patients had received any systemic treatment for 6 weeks before the commencement of the study.

THE USE OF topical antiandrogens in the treatment of acne has been intensively investigated ever since the pilosebaceous unit was found to be androgen dependent. In 1969, Cunliffe et al¹ used topical cyproterone acetate dispensed in dimethyl sulfoxide in 12 patients but found no improvement because of the lack of a suitable vehicle for the steroids. Seven years later, Pye et al² used 1% cyproterone acetate suspended in cetomacrogol cream BPC (formula A), but none of the patients showed any marked acne improvement. Meanwhile, a wealth of experience in the area of acne treatment has been gained by various clinicians and researchers. There are several alternatives to cyproterone acetate. Estrogens, progesterone, spironolactone, flutamide, and gonadotropin-releasing hormone analogues have all been used in various concentrations in clinical trials.⁷⁻⁹ These substances reduce sebaceous gland activity leading to a clinical improvement of acne. However, they have 1 problem in common. They are ingested and thus affect the entire human organism, producing systemic adverse effects.

It is well known that oral estrogens effectively suppress sebum excretion and improve acne in both men and women. However, the doses required to produce a therapeutic effect are associated with unacceptable adverse effects.¹⁰ The effect of topical progesterone also has been evaluated; whereas a reduction in the sebum excretion rate was demonstrated in female patients, no change was seen in male patients. Moreover, the sebosuppressive effect was lost after 3 months.¹¹

Antiandrogens show the most likely source of therapeutic success in the hormonal manipulation of the sebaceous gland.¹² Cyproterone acetate is most often prescribed in combination with ethinyl estradiol, with the maximum clinical effect generally seen between the third and sixth months of treatment. Oral spironolactone also has been shown both to decrease sebum excretion rates and to improve clinical acne.¹³ However, the prescription of spironolactone in this indication has been markedly reduced since the publication of animal research data indicating that it may cause breast cancer in rats.¹⁴ The oral nonsteroidal antiandrogen flutamide has been found to reduce acne but it is not suitable for clinical use.¹⁵ Chlormadinone acetate is a 19-norprogesterone with antiandrogenic properties. It is

used in combination with ethinyl estradiol as an oral contraceptive and has proved to be successful in cases of mild acne and seborrhea.¹⁶ Dienogest, apparently the first 19-nortestosterone derivative with antiandrogenic effects, is currently used in oral contraceptives and has been suggested for antiandrogen treatment. However, clinical trials evaluating the antiandrogen property of this substance are inadequate.¹⁷

Finally, the new topical nonsteroidal antiandrogen inocoteron acetate has produced only modest clinical effects in the treatment of acne.¹⁸ Evidence suggests that not all patients with acne exhibit elevated serum androgen levels.¹⁹ Rather, several studies have indicated an increased local formation of androgens, disturbances of the androgen metabolism,²⁰ or an increased sensitivity of the androgen receptor to normal levels of androgens.²¹ Consequently, antiandrogens and 5 α -reductase inhibitors may also play a role in the treatment of clinically hyperandrogenic women.²²

Despite the lack of success with topical cyproterone acetate in previous studies, we undertook a placebo-controlled trial using a liposome lotion as a carrier for cyproterone acetate to overcome the difficulty of delivering the active substance to the target cell. In the present study, the therapeutic effectiveness of topically applied cyproterone acetate in cases of acne was clearly demonstrated. Mean facial acne grades and lesion counts decreased significantly with both topical and oral cyproterone acetate. The preliminary serum determinations confirmed our initial expectation that topical use would result in lower serum cyproterone acetate levels than would oral cyproterone acetate intake. In fact, cyproterone acetate levels were 10 times lower after topical than after oral application, while producing a similar clinical response. According to data in the literature, cyproterone acetate levels after oral intake of 2 mg of cyproterone acetate can be expected to be an order of magnitude higher (4073 pg/mL); however, this is because a contraceptive effect is required.²³ Further studies and pharmacokinetic investigations are necessary to determine the optimal dosage and carrier regimens.

The mechanisms responsible for the effectiveness of topical cyproterone acetate are outside the scope of the present study. Whether topical cyproterone acetate acts directly on the skin or whether the serum levels, although low, are responsible for its therapeutic effects will have to be determined in further studies.

Notably, a significant therapeutic improvement compared with the placebo group was seen after only 3 months of treatment with both oral and topical cyproterone acetate. In summary, the results of this study suggest that topical application of the antiandrogen cyproterone acetate in the treatment of acne is as effective as oral cyproterone acetate in combination with ethinyl estradiol, provided that a suitable carrier is used. The liposome lotion we used unlocks the potential of topical cyproterone acetate application and releases the benefits of reduced adverse effects, at least within the treatment period evaluated in this study. Thus, the cyproterone acetate lotion represents a suitable alternative or additional local treatment for women with acne.

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Treatment of Acne Vulgaris

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THE MANAGEMENT OF ACNE VULGARIS by nondermatologists is increasing.¹ In this article we attempt to answer the question: what treatments in acne vulgaris have proven efficacy and how are these treatments best administered and individualized to optimize results and minimize complications? We considered the efficacy and safety of topical retinoids, topical antimicrobials, systemic antibiotics, hormonal treatments for women, and oral isotretinoin.

METHODS

A librarian-assisted literature search was performed for English-language randomized clinical trials. We used MEDLINE and EMBASE to identify all therapeutic clinical trials, meta-analyses, and systematic analyses concerning acne vulgaris from 1966 to 2004. We further cross-referenced bibliographies of identified articles. This search strategy identified 248 articles. We then evaluated titles and abstracts, and excluded studies that were not blinded, were not randomized, had sample sizes of fewer than 50, did not provide adequate information with respect to objective outcomes measures, contained no original data, pertained to treatments that are not available, did not involve humans, or were therapeutic failures. We used the following search words: *acne vulgaris*, *acne*, *tretinoin*, *tazarotene*, *ada-*

Context Management of acne vulgaris by nondermatologists is increasing. Current understanding of the different presentations of acne allows for individualized treatments and improved outcomes.

Objective To review the best evidence available for individualized treatment of acne.

Data Sources Search of MEDLINE, EMBASE, and the Cochrane database to search for all English-language articles on acne treatment from 1966 to 2004.

Study Selection Well-designed randomized controlled trials, meta-analyses, and other systematic reviews are the focus of this article.

Data Extraction Acne literature is characterized by a lack of standardization with respect to outcome measures and methods used to grade disease severity.

Data Synthesis Main outcome measures of 29 randomized double-blind trials that were evaluated included reductions in inflammatory, noninflammatory, and total acne lesion counts. Topical retinoids reduce the number of comedones and inflammatory lesions in the range of 40% to 70%. These agents are the mainstay of therapy in patients with comedones only. Other agents, including topical antimicrobials, oral antibiotics, hormonal therapy (in women), and isotretinoin all yield high response rates. Patients with mild to moderate severity inflammatory acne with papules and pustules should be treated with topical antibiotics combined with retinoids. Oral antibiotics are first-line therapy in patients with moderate to severe inflammatory acne while oral isotretinoin is indicated for severe nodular acne, treatment failures, scarring, frequent relapses, or in cases of severe psychological distress. Long-term topical or oral antibiotic therapy should be avoided when feasible to minimize occurrence of bacterial resistance. Isotretinoin is a powerful teratogen mandating strict precautions for use among women of childbearing age.

Conclusions Acne responses to treatment vary considerably. Frequently more than 1 treatment modality is used concomitantly. Best results are seen when treatments are individualized on the basis of clinical presentation.

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palene, *clindamycin*, *erythromycin*, *tetracycline*, *azelaic acid*, *benzoyl peroxide*, *minocycline*, *doxycycline*, *trimethoprim-sulfamethoxazole*, *flutamide*, *spironolactone*, *cyproterone-acetate*, *oral contraceptives*, *isotretinoin*, *clinical trials*, *review*, *therapy*, *treatment*, and *randomized controlled trials*.

We identified 29 randomized double-blind trials, which comprise the focus of this article. Where possible, data concerning responses to treatment were put in terms of percent reduction of inflammatory lesions, noninflammatory lesions (comedones), and total lesions.

A recent methodological literature review of acne therapy trials over the last 50 years found that methods of grading acne severity and methods of assessing outcome measures are highly incon-

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sistent.² There are more than 25 methods of assessing acne severity and more than 19 methods for counting lesions. Our literature review verifies the lack of standardized methodology. Nevertheless, analysis of acne therapy data does allow conclusions to be drawn that can direct therapeutic decisions.

In addition to the randomized controlled trials (RCTs), we reviewed selected articles that included data collected or analyzed after the trial, including meta-analyses and other systematic reviews. We also mention selected non-RCTs when they represent best evidence concerning established therapies that have not yet been studied in well-designed RCTs.

Pathophysiology

The origin of acne vulgaris is complex and incompletely understood. At least 4 pathophysiologic events take place within acne-infected hair follicles: (1) androgen-mediated stimulation of sebaceous gland activity, (2) abnormal keratinization leading to follicular plugging (comedo formation), (3) proliferation of the bacterium *Propionibacterium acnes* within the follicle, and (4) inflammation. In addition to these 4 basic mechanisms, genetic factors,³ stress,⁴ and possibly diet may influence the development and severity of acne.⁵

TREATMENT OF ACNE VULGARIS

Topical Retinoids

Retinoids, first shown in the 1970s to be of value for treating acne, are derivatives of vitamin A that prevent comedone formation by normalizing desquamation of follicular epithelium. The 3 main topical retinoids are tretinoin, adapalene, and tazarotene.

Tretinoin has long been considered the gold standard with which new products are compared. A meta-analysis of 5 multicenter randomized investigator-blinded trials involving 900 patients⁶ confirmed that total lesion counts reduced by 53% with tretinoin 0.05% gel and 57% with adapalene 0.1% gel (TABLE 1). Adapalene gel causes less irritation than tretinoin 0.05% gel, 0.1%

microsphere gel, or 0.05% cream.⁶⁻⁹ Tazarotene 0.1% gel had proven efficacy in an RCT showing 52% total acne reduction of total lesions compared with 33% with vehicle.¹⁰ Tretinoin was compared with tazarotene in a 12-week RCT with 169 patients.¹¹ Tazarotene 0.1% gel produced reductions in acne severity of 36% vs 26% with tretinoin 0.1% gel ($P = .02$). In another comparison trial, tazarotene 0.1% gel was more effective than tretinoin 0.025% gel in reducing noninflammatory lesion counts (55% vs 42%; $P = .042$) and equally effective in reducing inflammatory lesions.¹² In a multicenter RCT, adapalene 0.1% cream demonstrated a 38% reduction in total lesion counts vs 20% with vehicle.¹³ In a 12-week RCT with 145 patients tazarotene 0.1% gel was significantly better than adapalene 0.1% gel in terms of mean reductions in overall disease severity (44% vs 24%; $P < .001$), noninflammatory lesion count (71% vs 48%; $P < .0001$), and inflammatory lesion count (70% vs 55%; $P = .0002$).¹⁴ Alternate-day application of tazarotene 0.1% gel was equally effective to daily adapalene 0.1% gel in a 15-week RCT¹⁵ (Table 1).

Tretinoin, is available as a gel (0.01% and 0.025%), cream (0.025%, 0.05%, and 0.1%), and liquid (0.05%). Cutaneous erythema, peeling, and edema with tretinoin are dose-related adverse effects. Adapalene 0.1% is available as a cream, gel, and solution, all with similar efficacy.¹⁶ Tazarotene is available as 0.1% cream or gel formulations.

In summary, all topical retinoids effectively reduce the number of comedones and inflammatory lesions in the range of 40% to 70% (Table 1). Adapalene is less likely to cause skin irritation and is better tolerated than tretinoin or tazarotene, but tazarotene appears to be most efficacious.

Topical Antimicrobials

Currently available topical antimicrobials include clindamycin, erythromycin, tetracycline, and benzoyl peroxide. Azelaic acid may also be considered within this group because it has demonstrated antibacterial activity against

intrafollicular *P. acnes*.¹⁷ Our discussion focuses on 5 well-designed, randomized, double-blind trials assessing the effectiveness of topical antibiotics in acne. Newer formulations have been studied most rigorously.

Original placebo-controlled RCTs with clindamycin and erythromycin showed a 46% to 70% reduction in inflammatory lesions¹⁸⁻²¹ (Table 1). In another RCT, an erythromycin-4%-zinc combination reduced inflammatory lesions by 85% vs a 46% reduction using 2% erythromycin alone ($P < .001$).²² Recent interest has centered around combinations of topical antimicrobials with benzoyl peroxide or retinoids. Support for combining erythromycin or clindamycin with benzoyl peroxide includes a randomized, 10-week, multicenter, single-blind trial that enrolled 492 patients in which treatment with the combination products used twice daily was more effective than benzoyl peroxide alone.²³ Additionally, a review of 3 clinical studies involving 1259 patients concluded that the combination of clindamycin 1% benzoyl-peroxide 5% was more effective than either drug used alone in reducing lesions and suppressing *P. acnes*.²⁴ In 2 RCTs 334 patients were treated once nightly with either a combination clindamycin-benzoyl peroxide gel, benzoyl peroxide alone, clindamycin alone, or vehicle²⁵ (Table 1). After 11 weeks, 66% of patients in the clindamycin and benzoyl peroxide group experienced a good or excellent response compared with 41% in the benzoyl peroxide group, 36% in the clindamycin group, and 10% in the vehicle group. A similar 16-week trial showed a 53% lesion reduction with clindamycin 1% benzoyl-peroxide 5% vs 28% with clindamycin alone ($P = .013$).²⁶

Combining topical antibiotics with topical retinoids is also effective. Adapalene gel 0.1% plus clindamycin 1% was studied in a 12-week RCT involving 249 patients with mild to moderate acne. A significantly greater reduction in total ($P < .001$), inflammatory ($P = .004$), and noninflammatory lesions ($P < .001$) was seen in the clindamycin-plus-adapalene group than in the

TREATMENT OF ACNE VULGARIS

Table 1. Clinical Trials in Topical Acne Therapy

Table 1. Clinical Trials in Topical Acne Therapy						Reduction in Lesions, %		
Source	No. of Patients	Study Type	Length of Treatment, wk	Type of Acne*	Treatment	Inflammatory	Noninflammatory	Total
Topical Retinoids								
Cunliffe et al, ⁶ 1998	900	Meta-analysis	12	Mild to moderate facial acne	Adapalene 0.1% gel Tretinoin 0.025% gel	52 51	58 52	57 53
Shalita et al, ¹⁰ 1999	446	Randomized, double-blind, placebo-controlled, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel Tazarotene 0.05% gel Vehicle	42 39 30	55 45 35	52 44 33
Leyden et al, ¹¹ 2002	169	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel Tretinoin 0.1% gel	56 46	60 38	...
Webster et al, ¹² 2001	143	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel Tretinoin 0.025% gel	54 44	55 42	...
Lucky et al, ¹³ 2001	237	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Adapalene 0.1% cream Vehicle	36 19	38 20	38 20
Webster et al, ¹⁴ 2002	145	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel Adapalene 0.1% gel	70 55	71 48	...
Leyden et al, ¹⁵ 2001	164	Randomized, double-blind, multicenter	15	Mild to moderate facial acne	Adapalene 0.1% gel Tazarotene 0.1% gel†	54 57	58 55	...
Topical Antimicrobials								
Becker et al, ¹⁸ 1981	358	Randomized, double-blind, placebo-controlled, multicenter	8	Mild to moderate acne	Clindamycin phosphate Clindamycin hydrochloride Vehicle	66 63 42
Dobson and Belknap, ¹⁹ 1980	253	Randomized, double-blind, multicenter, placebo-controlled	12	Mild to moderate acne	Erythromycin 1.5% solution Vehicle	70 5	26 55	40 30
Leshner et al, ²⁰ 1985	225	Randomized, double-blind, multicenter, placebo-controlled	12	Mild to moderate acne	Erythromycin 2% Vehicle	46 19
Jones and Crumley, ²¹ 1981	156	Randomized, double-blind	12	Moderate to severe facial acne	Erythromycin 2% Vehicle	51 33
Habbema et al, ²² 1989	122	Randomized, double-blind, multicenter	12	Moderate to severe facial acne	Erythromycin-4%-zinc solution Erythromycin 2% lotion	85 46	68 49	...
Lookingbill et al, ²⁵ 1997	334	Randomized, double-blind, placebo-controlled, multicenter	11	Mild to moderate facial acne	Clindamycin-1%/BP 5% gel Clindamycin-1% gel BP 5% gel Vehicle	61 35 39 5	36 9 30 0	...
Cunliffe et al, ²⁶ 2002	79	Randomized, double-blind	16	Mild to moderate facial acne	Clindamycin-1% plus/BP 5% gel Clindamycin-1%	53 28
Oral and Topical Treatments								
Katsambas et al, ³³ 1989	92	Randomized, double-blind, placebo-controlled	12	Moderate acne	Azelaic acid 20% Placebo	72 47	56 0	...
Hjorth and Graupe, ³⁴ 1999	333 261	Randomized, double-blind, multicenter	20 24	Moderate to severe acne; Moderate to severe acne	Azelaic acid 20% Oral tetracycline Azelaic acid 20% Oral tetracycline	83 86 79 79

Abbreviation: BP, benzoyl peroxide; ellipses, data were not reported in the trial.

*For an example of acne severity, see the Figure.

†Therapy is taken on alternate days.

clindamycin-plus-vehicle group.²⁷ Other trials with clindamycin-tretinoin and erythromycin-tretinoin have shown similar results.²⁸⁻³²

Azelaic acid 20%, in an RCT that enrolled patients with moderate acne resulted in a 72% reduction of inflammatory lesions vs 47% with placebo.³³ Two RCTs compared oral tetracycline with topical azelaic acid 20%.³⁴ Reductions in inflammatory lesion counts were 83% for azelaic acid and 86% for oral tetracycline in one study and 79% for both drugs in another (Table 1). The efficacy of azelaic acid in mild to moderate acne matches that of tretinoin 0.05%, benzoyl peroxide 5%, or topical erythromycin 2%.¹⁷

Adverse effects of topical antibiotics include erythema, peeling, dryness, and burning.³⁵ Benzoyl peroxide can also cause an irritant dermatitis and bleach hair, clothes, and bed linens. A recent consensus has recommended that topical antibiotics should not be used alone due to the potential for bacterial resistance and relatively slow onset of action.³⁵ Antimicrobial resistance with benzoyl peroxide or azelaic acid has not been reported. Combining antibiotics with benzoyl peroxide is the most common practice. A minimum of 6 to 8 weeks of treatment is recommended.³⁵

Oral Antibiotics

Systemic antibiotics used in acne vulgaris have both antimicrobial and anti-inflammatory properties. They reduce *P. acnes* within follicles, thereby inhibiting production of bacterial-induced inflammatory cytokines.³⁶ Tetracycline and erythromycin suppress leukocyte chemotaxis³⁷ and bacterial lipase activity³⁸ while minocycline and doxycycline inhibit cytokines and matrix metalloproteinases thought to contribute to inflammation and tissue breakdown.³⁹ The main systemic antibiotics used in acne vulgaris are tetracycline, doxycycline, minocycline, and erythromycin.

Relatively few RCTs have studied the use of oral antibiotics in treating acne. A 12-week RCT involving 200 patients⁴⁰ showed a reduction in inflammatory lesions by 64% with tetracy-

cline vs 67% with erythromycin and a reduction in noninflammatory lesion counts by 34% with tetracycline vs 22% with erythromycin (TABLE 2). In another comparison trial topical clindamycin 1% showed a 72% reduction vs a 57% reduction using oral tetracycline and a 12% reduction with placebo.⁴¹

Doxycycline was recently studied in a RCT in which 51 patients received either a submicrobicidal dose (20 mg twice daily) for 6 months or placebo. Mean reduction in total lesions was 52% with doxycycline vs 18% with placebo ($P < .01$; Table 2).⁴² Even low doses of doxycycline may be effective by inhibition of collagenases including matrix metalloproteinases.³⁹ Doxycycline is frequently dosed at 100 mg/d for acne treatment although best evidence for those doses comes from small studies.⁴³

The efficacy of minocycline was assessed in a Cochrane review,⁴³ which concluded that minocycline is an effective therapy for moderate acne, but its efficacy compared with other acne therapies could not be reliably determined due to methodological flaws in the comparative trials. In a 3-month double-blind RCT, minocycline was somewhat more effective in reducing inflammatory lesion counts compared with zinc gluconate (67% vs 50%; $P < .001$).⁴⁴ Antimicrobial effects against *P. acnes* are greater with minocycline than with doxycycline or tetracycline,⁴⁵ and higher lipid solubility favors its bioavailability in pilosebaceous units.

Oral tetracycline is usually prescribed at a dosage of 500 mg twice a day. The absorption of tetracycline is reduced by food and dairy products; therefore, it must be taken on an empty stomach. Adverse effects include gastrointestinal tract dyspepsia, vaginal candidiasis in women, and a small risk of photosensitivity. In children younger than 10 years, tetracycline can cause enamel hypoplasia and a yellowish discoloration of the forming teeth.⁴⁶ Doxycycline has traditionally been used at a dose of 50 to 100 mg twice daily. Success with 20 mg/d may change clinical practice over time.⁴² Doxycycline causes gastrointestinal tract upset and is more

likely than tetracycline to cause photosensitivity.⁴⁶ Doxycycline can be taken with food. Tetracyclines should not be taken immediately before sleep because the pills may lodge in the esophagus and cause ulceration.

Minocycline is prescribed in a dosage range of 50 to 100 mg twice daily. Adverse effects include vertigo, dizziness, ataxia, and rarely a bluish discoloration of the skin.⁴⁶ Minocycline has also been reported to be associated with drug-induced lupus, autoimmune hepatitis, and a hypersensitivity syndrome.⁴⁷ The relative risk of developing a lupuslike syndrome with minocycline is 8.5 (95% confidence interval [CI], 2.1-35.0) compared with 1.7 (95% CI, 0.4-8.1) for other tetracyclines.⁴⁸

Antibiotic-resistant strains of *P. acnes* have increased steadily since the 1970s and are now found in more than 50% of cases in Europe and the United Kingdom.⁴⁹ Resistance of *P. acnes* to oral antibiotics is associated with treatment failures.⁵⁰ The effect of resistance to *P. acnes* with topical antimicrobial use is unclear.⁵¹ Resistance to tetracyclines is less common than to erythromycin⁴⁹ and is least with minocycline.⁵²

Recommendations for reducing antibiotic resistance in acne have been published recently and include using combined topical therapy—such as retinoids, benzoyl peroxide, or both when using topical antibiotics—and avoiding long-term use of topical or oral antibiotics when feasible.³⁵

Hormonal Therapy

Hormonal treatments for acne are tolerated in women only. These treatments, which decrease androgen expression, are based on the requirement for androgens in the pathophysiologic development of acne.⁵³⁻⁵⁴ A direct relationship between levels of circulating androgens and acne severity has not been established although prior studies suggest some degree of hyperandrogenemia in women with acne.⁵⁵⁻⁵⁷

Antiandrogenic compounds include oral contraceptives (OCs) and androgen-receptor blockers such as flutamide, spironolactone, and cyproter-

one acetate. Several OCs are now approved for use in acne. All contain 35 µg of estrogen or less. None of the androgen-receptor blockers are approved by the US Food and Drug Administration for use in the treatment of acne.

Oral contraceptives suppress ovarian androgens and reduce bioavailable testosterone by an estrogen-mediated in-

crease in steroid hormone binding globulin. After 6 months, 2 multicenter RCTs involving 507 women with moderate acne found that triphasic norgestimate and ethinyl estradiol (EE, Orthotri-cyclin [Ortho-McNeil Pharmaceutical Inc, Raritan, NJ]) had decreased inflammatory lesions by approximately 50% compared with a 30% re-

duction with placebo.^{58,59} Two RCTs studying the efficacy of 20 µg of EE plus 100 µg of levonorgestrel (Alesse [Wyeth, Madison, NJ]) showed total acne improvement of 23% to 40% compared with 9% to 23% with placebo (Table 2).^{60,61} A recent RCT involving 128 women showed an acne-lesion count reduction of 63% using the combination

Table 2. Clinical Trials in Oral Acne Therapy

Table 2. Clinical Trials in Oral Acne Therapy						Reduction in Lesions, %		
Source	No. of Patients	Study Type	Length of Treatment, wk	Type of Acne	Treatment	Inflammatory	Noninflammatory	Total
Antibiotics								
Gammon et al, ⁴⁰ 1986	200	Randomized, double-blind, multicenter	8	Moderate to severe acne	Oral erythromycin	67	22	...
					Oral tetracycline*	64	34	...
Braathen, ⁴¹ 1984	87	Randomized, double-blind	8	Moderate to severe acne	Oral tetracycline, 500 mg twice per d	57
					Clindamycin 1%	72		
					Placebo	12		
Skidmore et al, ⁴² 2003	51	Randomized, double-blind placebo-controlled, MC	24	Moderate facial acne	Oral doxycycline, 20 mg twice per day	50	54	52
					Placebo	30	11	18
Dreno et al, ⁴⁴ 2001	332	Randomized, double-blind multicenter	12	Moderate acne	Oral minocycline, 100 mg/d	67
					Zinc gluconate, 30 mg/d	50		
Oral Contraceptives								
Lucky et al, ⁵⁸ 1997	257	Randomized, double-blind placebo-controlled, multicenter	24	Moderate acne in women	Ethinyl estradiol, 35 µg plus norgestimate, 180 µg, 215 µg, or 250 µg of	62	...	53
					Placebo	39		27
Redmond et al, ⁵⁹ 1997	250	Randomized, double-blind placebo-controlled, multicenter	24	Moderate acne in women	Ethinyl estradiol, 35 µg plus norgestimate, 180 µg, 215 µg, or 250 µg of	51	...	46
					Placebo	35		34
Thiboutot et al, ⁶⁰ 2001	350	Randomized, double-blind placebo-controlled, multicenter	24	Moderate acne in women	Ethinyl estradiol, 20 µg plus levonorgestrel 100 µg	47	25	40
					Placebo	33	14	23
Leyden et al, ⁶¹ 2002	371	Randomized, double-blind, placebo-controlled	24	Moderate acne in women	Ethinyl estradiol, 20 µg plus levonorgestrel, 100 µg	32	13	23
					Placebo	22	4	9
Van Vloten et al, ⁶² 2002	128	Randomized, double-blind, multicenter	36	Mild to moderate acne in women	Ethinyl estradiol, 30 µg plus drospirenone, 3 mg	74	50	63
					Ethinyl estradiol, 35 µg plus cyproterone acetate, 2 mg	75	60	59
Isotretinoin								
Jones et al, ⁸¹ 1983	76	Randomized, double-blind	16	Moderate to severe acne	Isotretinoin, 0.1 mg/kg per d	80
					Isotretinoin, 0.5 mg/kg per d	80		
					Isotretinoin, 1.0 mg/kg per d	89		
Strauss et al, ⁸² 1984	150	Randomized, double-blind multicenter	20	Severe acne	Isotretinoin, 0.1 mg/kg per d	79
					Isotretinoin, 0.5 mg/kg per d	79		
					Isotretinoin, 1.0 mg/kg per d	89		
Strauss et al, ⁸³ 2001	600	Randomized, double-blind multicenter	20	Severe nodular acne	Isotretinoin, 1.0 mg/kg per d	90
					Micronized isotretinoin, 0.4 mg/kg per d	87		

Ellipses indicate that data were not reported in the trial.

*Variable doses used.

drugs of 35 µg of EE plus 3 mg of drospirenone (Yasmin [Berlex, Montreal, Quebec]) and a 59% reduction using 35 µg of EE plus 2 mg of cyproterone acetate (Diane-35 [Berlex]).⁶² Neither Alesse nor Yasmin is marketed for acne although both are used extensively for that indication.

Outside of the United States, the OC containing 35 µg of EE plus 2 mg of cyproterone acetate is the combination to which newer OCs have usually been compared for acne treatment. The progestin, cyproterone is an effective androgen-receptor blocker when used at higher doses in men with prostate cancer⁶³ and in women with acne, hirsutism, and polycystic ovary syndrome.⁶⁴ Best evidence for the use of this combination for acne comes from open studies or comparison trials with newer OCs containing levonorgestrel, drospirenone, and desogestrel. At least 60% improvement was demonstrated with all the above OCs.^{62,65,66} In Europe, the antiandrogen-progestin chlormadinone has been combined with EE in an oral contraceptive (Belara [Grunenthal, Aachen, Germany]) and has been shown to be superior to an OC containing levonorgestrel in treating acne.⁶⁷

Safety profiles are reasonable for OCs containing 35 µg of EE or less. Cardiovascular risks are not significantly increased in nonsmokers,⁶⁸ and breast cancer risks have not been shown to be increased overall.⁶⁹ The risk of deep-vein thrombosis increases from 1 per 10000 woman-years to 3.4 per 10000 woman-years during the first year and decreases thereafter.⁷⁰ Contraindications to using OCs in an otherwise healthy woman include smoking, migraine headaches with aura, and hypertension.⁷¹

Androgen-receptor blockers used in acne include spironolactone, flutamide, and cyproterone acetate. Spironolactone is well established as an aldosterone-blocking agent at doses of 25 mg/d in patients with heart failure.⁷² Higher doses (50-100 mg/d) are required for androgen-receptor blockade. Cyproterone acetate, in addition to being used as the progestin in the OC Diane-35, is

used in doses of 50 to 100 mg/d in women with hirsutism (not available in the United States). Flutamide, a nonsteroidal androgen-receptor blocker commonly used in prostate cancer is used in women with hirsutism and acne at doses of 250 to 500 mg/d.

Best evidence for the use of spironolactone in acne comes from 4 studies in which spironolactone alone or as an adjunct in doses of 50 to 200 mg/d showed 50% to 70% improvement of acne.⁷³⁻⁷⁶ A randomized comparison study of 53 participants showed a 50% improvement in acne and seborrhea among those who received a combination of 100 mg/d of spironolactone with an OC vs an 80% improvement among those who received 250 mg of flutamide with an OC.⁷⁷ Together with OCs, cyproterone acetate 50 to 100 mg/d is also effective in treating acne.^{78,79} Cyproterone acetate is, however, most commonly used in the low-dose formulation (2 mg) as part of an oral contraceptive.

Isotretinoin

Isotretinoin, a naturally occurring metabolite of vitamin A, inhibits sebaceous gland differentiation and proliferation, reduces sebaceous gland size, suppresses sebum production, and normalizes follicular epithelial desquamation. Isotretinoin is indicated in severe nodular acne and acne unresponsive to other therapies. It is used at a dosage of 0.5 to 1 mg/kg per day with a cumulative dosage of 120 to 150 mg/kg over a 4- to 6-month treatment period.

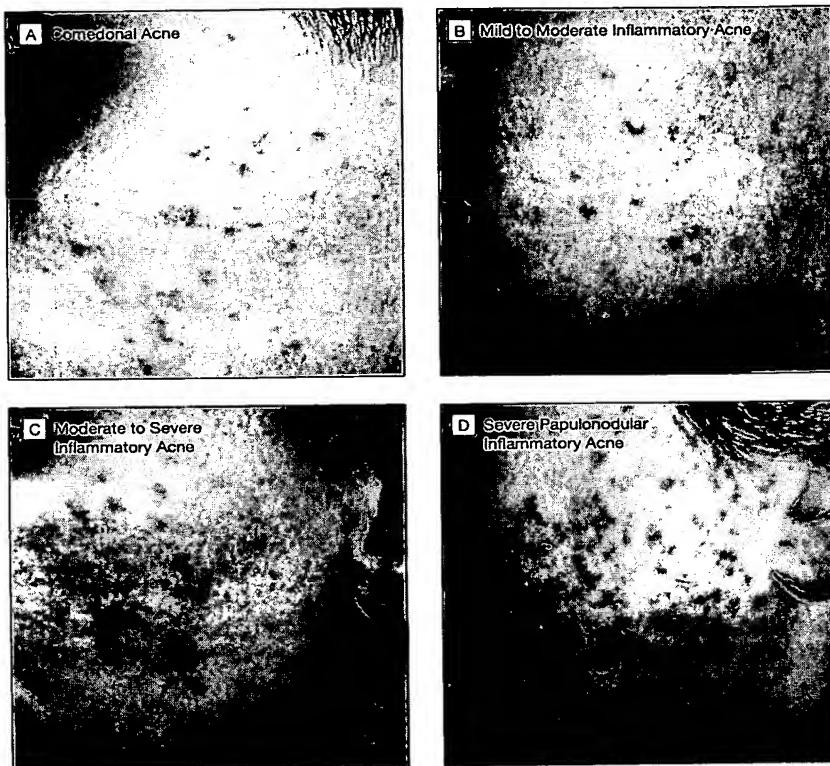
Isotretinoin was first shown to be effective in a nonrandomized clinical trial at an average dose of 2 mg/kg per day for 4 months in 14 patients with severe acne.⁸⁰ Complete clearing occurred in 13 of 14 patients and all 14 had prolonged remissions. A dose-response RCT involving 76 patients showed that at 4 months, total acne lesions were reduced by 80% with a treatment of 0.1 mg/kg per day or 0.5 mg/kg per day and by 89% with 1.0 mg/kg per day.⁸¹ A significantly greater treatment failure rate (45%) was observed with the lowest dose (0.1 mg/kg per day dosage). A related dose-comparison trial in 150 patients

found that retreatment was required in 42% of patients receiving 0.1 mg/kg per day and only 10% of patients receiving 1 mg/kg per day (Table 2).⁸² A new micronized formulation of isotretinoin (0.4 mg/kg per day) was equivalent in efficacy and safety to standard isotretinoin (1 mg/kg per day).^{83,84}

A 10-year follow-up of 88 patients who received isotretinoin in an initial dose of 0.5 or 1 mg/kg per day showed that 23% required a second course of isotretinoin,⁸⁵ usually within 3 years of stopping therapy. The daily and cumulative dosage was an important factor in determining relapse rate. Patients receiving 0.5 mg/kg per day had a relapse rate of 39% vs 22% in those taking 1 mg/kg per day ($P < .05$). A cumulative dosage of less than 120 mg/kg had a significantly higher relapse rate than those given a larger dose (82% vs 30%, respectively; $P < .01$). A recent chart review of 179 patients who had received 1 course of isotretinoin revealed that at the 3-year follow up, 35% had no recurrence; 16% required topical therapy; 27% required the use of oral antibiotics, and 23% required more isotretinoin.⁸⁶

Adverse effects of isotretinoin include dry lips, dry skin, dry eyes, decreased night vision, headache, epistaxis, and backache. Less common adverse effects include benign intracranial hypertension, so therapy must be stopped if a patient experiences persistent headaches. Isotretinoin can also be associated with a mild to moderate elevation in liver enzymes and in serum lipid indices, especially triglycerides.⁸⁷ It is generally well accepted that baseline cholesterol, fasting triglycerides, and liver function tests be done. Follow-up tests are recommended at weeks 4 and 8. If these test results are normal, further testing at week 12 may not be necessary.

Isotretinoin is a proven teratogen, and its use necessitates adequate contraception during and 6 weeks after therapy, as well as baseline and monthly pregnancy tests. Major malformations occur in 40% of infants exposed to isotretinoin in the first trimester.⁸⁸ It is strongly recommended that patients have 2 negative pregnancy tests be-

Figure. Severity and Type of Acne

fore starting isotretinoin and regular monthly pregnancy tests thereafter. Current prescribing regulations in the United States require physicians to identify on each prescription that patients have met the above qualifications and have signed a consent form. Further measures are being discussed to mandate a single, centralized registration and tracking system for all health care professionals involved with isotretinoin. A recent evidence-based review examined the issue of an increasing number of reported cases of depression and suicide associated with isotretinoin.⁸⁹ Epidemiological evidence for an association between isotretinoin and depression is currently lacking.⁸⁹ Furthermore, there is a 24.7% and 13.3% prevalence of anxiety and depression, respectively, in patients with acne.⁹⁰ Until well-designed studies are conducted, patients and their relatives must

be informed about depressive symptoms, and screening for depression should be an essential part of each visit.

CASE-BASED CLINICAL APPLICATIONS

Diagnosis

The diagnosis of acne vulgaris is usually uncomplicated. Differential diagnoses mainly include rosacea, perioral dermatitis, bacterial folliculitis, and drug-induced acneiform eruptions. The presence of comedones confirms the diagnosis of acne vulgaris.

Evidence-based literature in acne treatment is growing, and there is sufficient evidence to justify specific treatments for most clinical presentations. Successful outcomes frequently require nuance in management and a thorough understanding of all treatment modalities. Good outcomes are based on what is perceived by the pa-

tient as well as what can be measured. Since morbidity in acne is primarily emotional (psychological), different degrees of success may satisfy different individuals. Acne severity fluctuates over time and treatments often need to change accordingly.

Comedones Only

For this treatment, topical retinoids are the mainstay of treatment. Choices include tretinoin, adapalene, and tazarotene (FIGURE, A). Treatment response expectations are in the range of a 40% to 70% reduction in number of comedones within 12 weeks.^{6,11,14} Creams and lower concentrations of retinoids are less irritating but may take longer for a response than higher concentrations and gels. Short-contact therapy, starting with 30 seconds and building up to 1 hour or more followed by washing, was demonstrated effective and safe in a study with tazarotene gel⁹¹ and could be considered with all topical retinoids. Application should be to the entire area of involvement. Maintenance treatment is usually required.

Inflammatory Acne (Papules and Pustules), Mild to Moderate Severity

Topical antibiotics are the treatment of choice for these patients (Figure, B). Choices include benzoyl peroxide, azelaic acid, clindamycin, erythromycin, and dual agents combining benzoyl peroxide with either erythromycin or clindamycin. Current recommendations favor combining topical antimicrobial products with topical retinoids if they can be tolerated by patients.^{27,35,92} Benzoyl peroxide, 2% to 10%, is an inexpensive and effective antimicrobial that is not associated with antimicrobial resistance.⁹³ The dual-agent products combining topical antibiotics (clindamycin, erythromycin) with benzoyl peroxide are more effective than antibiotics alone.^{23-25,93} Best results require 8 to 12 weeks and maintenance therapy is usually required. Reasonable response expectations are in the range of 30% to 80%.^{17-20,23,26}

Moderate to Severe Inflammatory Acne

Oral antibiotics including the tetracyclines (minocycline, doxycycline, tetracycline) are the first-line choices (Figure, C). Erythromycin is recommended less often because of its association with resistant *P. acnes*.⁹⁴ Trimethoprim-sulfamethoxazole has been reported to be successful, but there is an unacceptably high risk of severe adverse events. Response expectations with oral antibiotics are in the range of 64% to 86%.^{34,40}

All oral antibiotics require a minimum of 6 to 8 weeks of treatment. There are no strict regulations on duration of use; but the recent increase in the prevalence of resistant organisms has resulted in current recommendations to encourage using antibiotics for shorter periods and to avoid the long-term use of antibiotics for maintenance therapy.³⁵

Severe Papulonodular Acne

Oral isotretinoin is indicated for severe papulonodular acne (Figure, D), treatment failures, scarring, or frequently relapsing acne or in cases where psychological distress is severe. Isotretinoin is used as a single-drug therapy except for women for whom concomitant OCs are strongly recommended. Best responses are seen with daily doses of 1 mg/kg per day for a period of 20 weeks or a total accumulative dose of 120 mg/kg.⁸⁵

A rare adverse effect of isotretinoin is called acne fulminans, characterized by extensive erosive lesions, fever, arthralgias, and leukocytosis. Treatment requires systemic corticosteroids. In a recent report of 25 cases of acne fulminans, best responses were seen with 0.5 to 1.0 mg/kg of prednisone daily for 4 to 6 weeks, with isotretinoin resumed on week 4, starting with 0.5 mg/kg per day and increasing gradually.⁹⁵

Women With Acne

Hormonal treatments with OCs or androgen-receptor blockers have been shown to be helpful and are reviewed elsewhere.⁹⁶ For a woman with acne who desires birth control, OCs are an excellent initial choice. Oral contra-

ceptives do not preclude using standard therapies if indicated. Approved OCs for use for acne include Orthotricyclin (in the United States and Canada), Estrostep (in the United States [Pfizer, New York, NY]), and Diane-35 (Canada). The results of RCTs and other best evidence, expected improvement with OCs alone is from 40% to greater than 70% (TABLE 3).

For those who do not respond to OCs, androgen-receptor blockers, alone or as adjuncts to OCs, have response expectation in the range of 50% to 80%. A treatment dosage of 50 to 100 mg/d of Spironolactone is well tolerated, with adverse effects including diuretic effect, breast tenderness, and menstrual irregularities if OCs are not used concomi-

tantly.⁹⁷ Another well-tolerated treatment is 250 mg/d flutamide. Its potential adverse effects include gastrointestinal tract upset and, at higher doses, hepatotoxicity. Periodic liver function tests are recommended with any dose of flutamide. Similar to spironolactone is 50 to 100 mg/d of cyproterone acetate. Hepatotoxicity has been reported rarely in men receiving cyproterone acetate for prostate cancer,⁹⁸ and in women receiving OCs containing cyproterone acetate.⁹⁹ Hormonal treatments for acne treatment are usually prolonged, depending on response and tolerance.

Laboratory Studies

For women with regular menstrual cycles, serum-androgen measurements

Table 3. Most Common Adverse Effects of Systemic Acne Medications

Drug	Approximate Frequency
Oral Antibiotics	
Dyspepsia, %	30
Photosensitivity	Rare (highest: doxycycline)
Benign intracranial hypertension	Rare
Hypersensitivity reaction	Rare
Lupuslike syndrome*	
Tetracyclines as a group	14.2 Cases per 100 000 prescriptions
Minocycline	52.8 Cases per 100 000 prescriptions
Isotretinoin, %	
Mucocutaneous (cheilitis)	95
Teratogenicity	25-40 of exposed fetuses
Hypertriglyceridemia	25
Elevation of liver transaminases	15
Hypercholesterolemia	7
Oral contraceptives, %	
Dysmenorrhea	10
Nausea	2-10
Breast tenderness	6
Headache	5
Depressed mood	3-30
Venous thromboembolism†‡	3.4 per 10 000 woman-years† Highest during first year of use
Spironolactone, %‡	
Diuretic effect	30
Dysmenorrhea	20
Dysphoria	20
Breast tenderness	18
Flutamide§	
Hepatotoxicity, %	1 (doses >500 mg)
Cyproterone acetate	
Hepatotoxicity	Rare (doses of 50-100 mg)

*Sturkenboom et al.⁴⁸

†Lidegaard et al.⁷⁰

‡Shaw et al.⁹⁷

§Lin et al.⁹⁸

||Rudiger et al.⁹⁹ and Legro.¹⁰⁰

are not necessary. For those with rapid onset of hyperandrogenism and virilization, an androgen-secreting ovarian or adrenal tumor can be excluded with a normal total testosterone and dehydroepiandrosterone sulfate levels, respectively. Irregular menses, hirsutism, obesity, or a family history of type 2 diabetes suggest a possible endocrinopathy, such as polycystic ovary syndrome. Further studies may be indicated, which could include measurement of gonadotropins, free testosterone, 17-hydroxy progesterone, prolactin, and androstenedione.^{57,100} Unfortunately, there is no widely accepted best laboratory test in this setting.¹⁰¹

Conclusion

Current treatments in acne target one or more of the known mechanisms involved in the disease. Combining more than 1 treatment frequently yields optimal responses. Patients may require adjustment of therapies depending on their degree of improvement and level of tolerance to the treatments.

Author Contributions: Drs Shaw and Haider had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Shaw, Haider.

Acquisition of data: Shaw, Haider.

Drafting of the manuscript: Shaw, Haider.

Critical revision of the manuscript for important intellectual content: Shaw, Haider.

Study supervision: Shaw.

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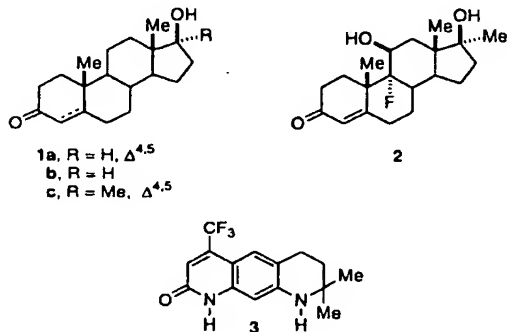
Discovery of a Potent, Orally Active, Nonsteroidal Androgen Receptor Agonist: 4-Ethyl-1,2,3,4-tetrahydro-6-(trifluoromethyl)-8-pyridono[5,6-*g*]-quinoline (LG121071)

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Introduction. Deficiencies in circulating levels of the androgens testosterone (T, **1a**) and dihydrotestosterone (DHT, **1b**) in hypogonadal men can be compensated for by administration of exogenous androgens,^{1,2} which have proven efficacious in hormone replacement therapy, abrogating age-related deterioration of muscle and bone,³ and regulating plasma lipids.⁴ Cancer cachexia,⁵ male contraception,⁶ and performance enhancement⁷ have also been investigated as clinical targets of androgen therapy.⁸



The beneficial effects of administered steroidal androgens are often overshadowed by their rapid metabolic conversion to DHT by 5 α -reductase and to estrogens by aromatase, resulting in side effects. Circumventing this metabolism through alternate routes of administration, such as intramuscular injection or transdermal patch applied to the scrotal skin,⁹ and attempts to improve oral half-life of T using long-chain alkyl esters as prodrugs have met with limited success.¹⁰ Alkylation of androgens at C-17, as in methyltestosterone (**1c**) and fluoxymesterone (**2**), has been observed to slow hepatic metabolism, allowing oral administration, but subsequent liver toxicity limits their use for chronic administration.

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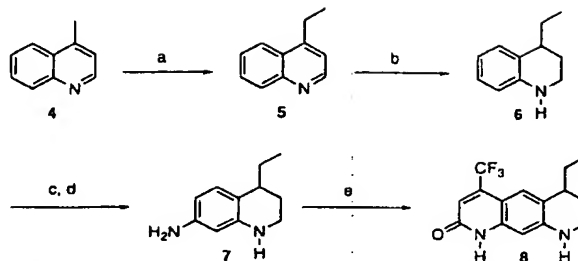
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Scheme 1^a



^a (a) LDA, MeI, THF, -78°C , 98%; (b) $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, NaBH_4 , MeOH, 0°C –rt, 96%; (c) HNO_3 , H_2SO_4 , -10°C , 10 min, 91%; (d) H_2 , 10% Pd/C, EtOH/EtOAc, rt, 16 h, 94%; (e) ethyl 4,4,4-trifluoroacetoacetate, ZnCl_2 , EtOH, reflux, 8 h, 82%.

Efforts to identify more receptor- and tissue-selective compounds which might avoid steroid-related side effects and toxicities have shifted focus away from steroid structural templates. Though representatives of several structural classes have been developed or are currently undergoing late-stage preclinical development as human androgen receptor (hAR) antagonists,¹⁰ efforts to discover and develop nonsteroidal AR agonists have been few.¹¹ To date, no known nonsteroidal AR agonists have been reported to exhibit activity in vivo. In the course of our investigations into the structure–activity relationships of dihydroquinoline-based AR antagonists such as **3**,¹² we had the opportunity to examine the effect of removal of 2,2-dialkyl substitution. It had previously been noted that substitution at this position played a critical role in driving the receptor into a transcriptionally inactive conformation and that lack of geminal substitution (as in 2-monoalkyl-substituted or 2,2-dihydro analogues) greatly impacted the transcriptional competency of the AR–ligand complex. It was more specifically observed that tetrahydro analogue **8** (LG121071) exhibited tighter binding affinity than the substituted analogues and scored as a full agonist in cotransfection assays, which led us to investigate the ability of **8** to exhibit AR agonist activity in a classic animal model.

Chemistry. Our previously reported efforts at construction of 2,2-dialkyl-substituted pyridonoquinolines involved sequential annulation of each terminal ring about a central core through cyclization strategies.¹³ Synthesis of 1,2,3,4-tetrahydro-8-pyridono[5,6-*g*]quinolines was achieved in a more efficient manner from a single annulation onto an existing quinoline core after appropriate functionalization (Scheme 1).¹⁴ The requisite intermediate 4-ethyl-1,2,3,4-tetrahydroquinoline (**6**)¹⁵ was synthesized in a two-step sequence starting from commercially available lepidine (**4**). Treatment of **4** with LDA¹⁶ and trapping of the resultant anion with iodomethane furnished 4-ethylquinoline (**5**)¹⁷ in excellent yield (98%). Reduction of the quinoline ring using NaBH_4 – NiCl_2 ¹⁸ afforded the required 4-ethyltetrahydroquinoline **6** in 96% yield. Standard nitration and subsequent reduction by catalytic hydrogenation provided diamine **7**, which smoothly underwent Knorr cyclization¹⁹ (ethyl 4,4,4-trifluoroacetoacetate, ZnCl_2 ,

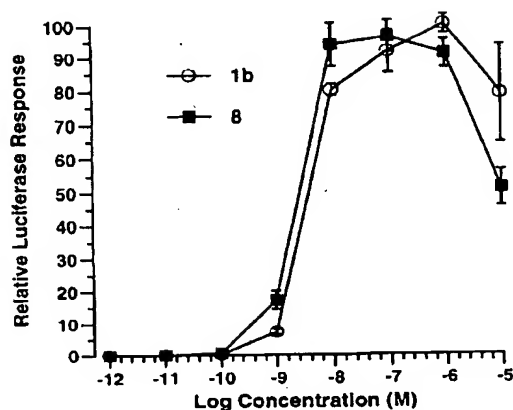


Figure 1. hAR agonist dose response of compound 8 in cotransfected CV-1 cells. Values represent mean \pm SEM of at least triplicate determinations.

Table 1. hAR Agonist and Antagonist Activity in Cotransfected CV-1 Cells and Binding Affinities for hAR in Transiently Transfected COS-1 Cells^a

compd	hAR agonist activity		hAR antagonist activity		hAR binding K_i^b (nM)
	EC ₅₀ ^b (nM)	efficacy ^c (%)	IC ₅₀ ^b (nM)	efficacy ^c (%)	
1b	5 \pm 1	100 \pm 0	na ^d		3 \pm 1
2	0.3 \pm 0.1	128 \pm 12	na		4 \pm 1
8	4 \pm 1	100 \pm 7	7481 \pm 1655	36 \pm 10	17 \pm 3

^a Cotransfection assay experiment values represent at least triplicate determinations. ^b Values represent mean \pm SEM. EC₅₀ values represent the concentration of ligand required to give half-maximal activation; IC₅₀ values represent the concentration of ligand required to give half-maximal inhibition of DHT at its EC₅₀. ^c Efficacies were compared to that of dihydrotestosterone (100%). ^d Not active; defined as efficacy < 20%, potency > 10 000 nM.

EtOH, reflux) to afford 4-ethyl-1,2,3,4-tetrahydro-6-(trifluoromethyl)-8-pyridono[5,6-*g*]quinoline (8).

In Vitro and in Vivo Biological Activity. 1. **Cotransfection and Binding Assays.** The AR agonist activities of 8 as well as that of the known AR agonists 1b and 2 were studied experimentally in a cellular background through both ligand-dependent stimulation of reporter gene (luciferase) induction using the cotransfection assay²⁰ (Figure 1) and a whole-cell receptor binding assay (Table 1). Also included in Table 1 are data for the compound and standards tested in cotransfection assays using hAR in the antagonist mode in the presence of DHT at its EC₅₀. Activities on other IRs including human progesterone receptor (hPR-B), human glucocorticoid receptor (hGR), human mineralocorticoid receptor (hMR), and human estrogen receptor (hER) were also determined, and there was found to be no agonist or antagonist response induced by compound 8.²¹

2. **Two-Week LH Suppression Assay in Rats.** The hypothalamic-pituitary axis in male rats and men functions as a feedback loop to regulate circulating levels of endogenous steroid (T, DHT) and gonadotropins [luteinizing hormone (LH), follicle-stimulating hormone (FSH)]. Castration causes a dramatic increase in secretion of pituitary LH, and restoration of androgen control and subsequently restoration of LH to normal physiological levels can be achieved by administration of

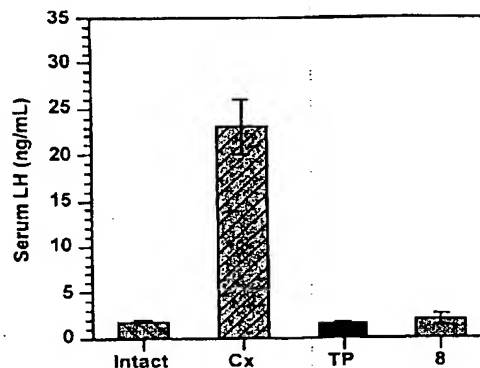


Figure 2. Suppression of luteinizing hormone (LH) in castrated (Cx) mature rats by compound 8 (20 mg/kg, po) or testosterone propionate (TP) (1 mg/kg, sc) daily for 2 weeks ($n = 4$ for all groups). All values represent the mean serum LH concentrations \pm SEM.

exogenous androgens.²² Compound 8 was examined in this established model of androgen action to assess its ability to suppress castration-induced elevation of serum LH after oral administration (Figure 2).

Results and Discussion. Compound 8 stimulates reporter gene expression in a concentration-dependent manner in the cotransfection assay, with a potency and efficacy equivalent to that of DHT (Figure 1). A slight antagonist response is observed only at the highest concentration (10 μ M) in the cellular background used for this assay. In vivo, testosterone propionate administered subcutaneously at a dose of 1 mg/kg completely blocked the effects of castration, restoring serum LH levels to that of the intact control animals. Compound 8 at a dose of 20 mg/kg administered orally was also fully efficacious at suppressing the castration-induced elevation of LH in the male rat.

Conclusion. The data shown for LG121071 (8) represent the discovery of the first known orally active, nonsteroidal AR agonist. This finding, together with earlier reports from these laboratories,²³ provides further support for a drug discovery approach targeting both agonists and antagonists of sex steroid hormone receptors diverging from a common pharmacophore. Compounds based on this novel template are the subjects of continued investigations toward development of therapeutically useful AR agonists with desirable tissue selectivity and avoiding structure-based side effects associated with compounds derived from steroidal templates.

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Supporting Information Available: Synthetic procedures and chemical characterization data for compounds 5–8 and biological assay methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Gonadal and Adrenal Androgens Are Potent Regulators of Human Bone Cell Metabolism In Vitro

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ABSTRACT

Androgens stimulate bone formation and play an important role in the maintenance of bone mass. Clinical observations suggest that both gonadal and adrenal androgens contribute to the positive impact of androgenic steroids on bone metabolism. We investigated the mechanism of action of the adrenal androgen dehydroepiandrosterone (DHEA) and its sulfated compound dehydroepiandrosterone sulfate (DHEAS) on human osteoblastic cells (HOCs) in vitro. The DHEA- and DHEAS-induced effects were analyzed in parallel with the actions elicited by the gonadal androgen dihydrotestosterone (DHT). There was no qualitative difference between the effects of gonadal and adrenal androgens on HOC metabolism in vitro. Both were stimulatory as regards cell proliferation and differentiated functions, but the gonadal androgen DHT was significantly more potent than DHEA. The actions of DHT and DHEA on HOC proliferation and alkaline phosphatase (ALP) production could be prevented by the androgen receptor antagonist hydroxyflutamide and inhibitory transforming growth factor β antibodies (TGF- β ab), respectively, but were not affected by the presence of the 3β -hydroxysteroid dehydrogenase (3β HSD) and 5α -reductase (5-AR) inhibitor 17β -N,N-diethylcarbamoyl-4-methyl-4aza- 5α -androstan-3-one (4-MA). This indicates that DHT and DHEA (1) exert their mitogenic effects by androgen receptor-mediated mechanisms, (2) stimulate ALP production by increased TGF- β expression, (3) that the action of DHT is not affected by the presence of 4-MA, and that (4) DHEA does not need to be metabolized by 3β HSD or 5-AR first to exert its effects on HOCs in vitro. (J Bone Miner Res 1997;12:464–471)

INTRODUCTION

THE GONADAL ANDROGENS TESTOSTERONE AND DIHYDROTESTOSTERONE (DHT) are important regulators of bone cell activity and bone mass in animals^(1,2) and humans.^(3,4) Additionally, declining serum levels of gonadal androgens are associated with osteopenia in vivo.⁽⁵⁾ Gonadal androgens are known to act directly on osteoblasts, stimulating growth and differentiation of osteoblastic cells in vitro by binding to an androgen receptor.^(6,7) Adrenal androgens also play a role in maintaining bone mass. Significant positive correlations have been observed between serum levels and bone mass in both animal and clinical studies.^(8,9) Dehydroepiandrosterone (DHEA), the major adrenal androgen, reduces the ovariectomy-induced osteopenia in rats⁽¹⁰⁾ and DHEA serum levels are positively correlated with bone

mass in aging women and patients with deficient adrenal androgen production.^(11–13) Although a recent study has demonstrated that gonadal and adrenal androgens stimulate alkaline phosphatase production by human bone cells by the same mechanism,⁽¹⁴⁾ the effects of adrenal androgens on other aspects of bone cell metabolism, and the mechanism by which they exert their effects on bone cells, remain unknown.

In view of the growing interest in DHEA and its sulfated metabolite (DHEAS) as anti-aging drugs,^(15–20) the present studies were carried out to determine the effects of the adrenal androgen DHEA on the growth and differentiated functions of human osteoblastic cells (HOCs) in both early and late stages of culture to identify a possible mechanism of action of adrenal androgens on HOC metabolism.

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MATERIALS AND METHODS

Human bone cell cultures

HOCs were obtained from bone biopsies of healthy, male patients (18, 19, 27, 43, 54, and 68 years) undergoing elective orthopedic surgery. The experimental protocol was approved by the local ethics committee of the University of Heidelberg. HBCs were harvested and characterized as HOCs as described previously.⁽²¹⁾ Briefly, cortical bone chips from the femoral shaft were thoroughly cleaned from periosteal tissue and bone marrow. After 10–20 days of continuous culture in Dulbeccos's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, U.S.A.) with 10% bovine calf serum (BCS) (Hyclone, Logan, Utah, U.S.A.) and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) solution (PS) (Irvine Scientific, Santa Ana, CA, U.S.A.), cells were digested from the bone chips by trypsinization and identified as bone cells on the basis of osteocalcin secretion, formation of mineral in vitro, type I collagen mRNA expression, and 1,25-dihydroxyvitamin D₃-inducible alkaline phosphatase production. HOCs were grown at 37°C in humidified air (5% CO₂/95% air) and only HOCs of the first and second passage were used in experiments. Twenty-four hours and 1 h prior to the addition of the steroids, the culture medium was changed to phenol red-free DMEM containing 1% charcoal-treated BCS and 1% PS. HOCs were enumerated with a hemocytometer after 48 and 72 h. Alkaline phosphatase (ALP) activity, cellular protein, the number of ALP positive staining (ALP⁺) HOCs, and osteocalcin secreted into the culture medium were determined after 72 h of continuous steroid treatment. For mineralization assays, HOCs were cultured in phenol red-free DMEM containing 10% charcoal-treated BCS and 1% PS. After reaching confluency, the culture medium was changed to fresh medium containing 10 nM β-glycerophosphate, 50 µg/ml ascorbic acid, and steroids. For all experiments, control cultures received ethanol vehicle (0.01% v/v) but did not receive the steroid. The adrenal androgens (DHEA, DHEAS) were generous gifts of Dr. Jan Stepan, Prague, Czech Republic; DHT was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) 17β-N,N-diethylcarbamoyl-4-methyl-4aza-5α-androstan-3-one (4-MA) was obtained from Dr. Rasmusson (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.).

Statistical significance between groups was analyzed by the two-tailed Student's *t*-test or two-way analysis of variance (ANOVA) as indicated. The results were expressed as percent of mean (*n* = 6) of controls (mean ± SD). All experiments were repeated at least twice.

DNA synthesis assay

[³H]thymidine incorporation was measured to detect the early effects of androgens on DNA synthesis. HOCs were cultured for 24 h in experimental medium in the presence of androgens, and [³H]thymidine was added at 1 µCi/well for the last 6 h of the incubation period. After incubation with [³H]thymidine, the medium was removed, the cell layer rinsed with phosphate buffered saline (PBS) twice, and

precipitated with 0.5 ml of 5% trichloroacetic acid (TCA). After rinsing with 5% TCA, the cells were solubilized using 0.5 ml 0.25 M NaOH. One-half milliliter NaOH cell lysates were transferred into a 3.5 ml scintillation cocktail and measured in a β-scintillation counter.

Specific ALP activity and ALP staining

After 72 h of continuous steroid treatment, cultures were rinsed with PBS, extracted with 0.01% Triton X-100 (Sigma Chemical Co.) containing 0.01% azide, 12.5 mM Tris buffer and 12.5 mM sodium bicarbonate at pH 10.3. The extracts were then frozen at –200°C overnight. The ALP activity was determined in the Triton X-100 cell extracts by spectrophotometrically measuring the breakdown of paranitrophenol-phosphate by ALP. The protein content in the Triton X-100 cell extracts was determined using the bicinonic acid (BCA) protein assay (Pierce, Rockford, IL, U.S.A.) and specific ALP activity was expressed as U/g of protein.

For ALP⁺ staining, HOCs were rinsed with PBS and stained using an azo-dye capture technique employing Naphthol AS-TR phosphate as substrate, fast blue violet LB salt, and 100 µl of 10 mM magnesium chloride in 0.05 M tris buffer, pH 8.6 (Sigma Chemical Co.). After stain removal and a second rinse with PBS, the stained cells were counted in rasters across the culture wells under an inverted Leitz microscope (Leitz, Wetzlar, Germany) equipped with an eyepiece reticule.

Osteocalcin secretion

The concentration of osteocalcin in the culture media was measured by radioimmunoassay (OSCAtest assay, Henning Berlin GmbH, Berlin, Germany). The amount of osteocalcin measured in the culture media was corrected for cell protein in Triton extracts of the culture wells.

Mineralization assay

HOC populations were cultured in mineralization medium for 7 days before adding 1 µCi/ml Ca⁴⁵ (ICN, Irvine, CA, U.S.A.). After 24 h, the culture wells were thoroughly rinsed twice with PBS and the mineral dissolved in 1 N HCl. The hydrolyzed mineral was centrifuged at 14,000 rpm for 5 minutes, and the amount of Ca⁴⁵ incorporated into mineral was measured by liquid scintillography in an aliquot of the supernatant.

Binding assay

To examine DHEA binding in confluent HOC populations, we performed displacement binding experiments in a whole-cell binding assay using the androgenic steroids DHT, methyltrienolone (= R1881), and DHEA. Briefly, 24 h prior to binding analysis of confluent HOC populations, the culture medium was changed to serum-phenol red-free DMEM. For the binding experiments, cultures were rinsed twice with cold PBS, and the culture medium was changed to serum-phenol red-free DMEM. Cultures were incubated with 1 nM of the radiolabeled androgenic

TABLE 1a. EFFECTS OF DHT AND DHEA ON HUMAN OSTEOBLASTIC CELL PROLIFERATION

	Concentration (nM)					
	0.001	0.01	0.1	1	10	100
48 h						
DHT	121 ± 31*	195 ± 21	226 ± 14	273 ± 32	339 ± 22	365 ± 44
DHEA	143 ± 12	200 ± 20	200 ± 14	243 ± 13	268 ± 31	256 ± 12
DHEA + OHFlu [†]	ND	127 ± 46 (NS)	154 ± 68 (NS)	145 ± 39 (NS)	154 ± 36 [‡]	145 ± 78 (NS)
72 h						
DHT	136 ± 42*	129 ± 9	139 ± 16	146 ± 21	200 ± 30	212 ± 14
DHEA	125 ± 6	141 ± 10	164 ± 12	180 ± 10	209 ± 22	235 ± 22

DHT and DHEA at the indicated concentrations increased HBC proliferation after 48 and 72 h. There was no significant effect on cell proliferation with either compound after 24 h of treatment (data not shown). After 48 h, DHT was significantly more mitogenic than DHEA as analyzed by ANOVA. The presence of 100 nM hydroxyflutamide (OHFlu) abolished the stimulatory effect of DHEA on human bone cell proliferation (data presented as mean of % of control ± SD; control counts: 575 ± 96, $p < 0.001$; [†] $p < 0.05$; NS, not significant; ND, not determined; [‡] effect of OHFlu alone 126 ± 36%).

TABLE 1b. EFFECTS OF DHEA AND DHT ON DNA SYNTHESIS

	Concentration (nM)				
	0	0.01	0.1	1	10
DHEA	881 ± 32*	924 ± 81 (NS)	973 ± 163 (NS)	1057 ± 96	1560 ± 69
DHEA + 1 nM DHT	1182 ± 164 [†]	1127 ± 91	1291 ± 165	1314 ± 216	1613 ± 182
DHT	1029 ± 122*	1046 ± 112 (NS)	1086 ± 31 (NS)	1318 ± 156	1394 ± 32
DHT + 1 nM DHEA	1336 ± 67 [‡]	1237 ± 199 (NS)	1335 ± 117	1674 ± 337	1674 ± 285

Human bone cells were treated with various doses of DHEA (DHT) in the absence and presence of 1 nM DHT (DHEA) for 24 h. [³H]thymidine incorporated into trichloroacetic acid precipitable material was counted by liquid scintillography as previously described.⁽¹⁶⁾

* Control counts measured in the absence of any steroid. [†] Counts measured after treatment with 1 nM DHT alone. [‡] Counts measured after treatment with 1 nM DHEA alone (data in cpm ± SD; $p < 0.05$; NS, not significant).

compound ³H-methyltrienolone (³H-R1881 obtained from New England Nuclear, Danvers, MA, U.S.A., specific activity 86 Ci/mmol) alone (= control) or together with various doses of unlabeled DHEA, DHT, or R1881 for 1 h at 4°C. The androgenic steroid methyltrienolone (R1881) was utilized for the whole-cell binding experiment because R1881 has a higher affinity to the androgen receptor (AR) than physiological compounds (e.g., testosterone) and cannot be metabolized by osteoblasts. After 1 h of incubation at 4°C, the cultures were rinsed twice with PBS, and 3 ml of 0.25 N NaOH were added. After solubilization of the cell layers, the cell lysates were added to 15 ml of scintillation fluid and evaluated in a β -scintillation counter.

RESULTS

Mitogenic action of gonadal and adrenal androgens

The observation of positive correlations between serum levels of adrenal androgens and bone mass⁽¹³⁾ and the finding that the number of differentiated osteoblasts determines the amount of newly formed bone⁽²²⁾ suggest that adrenal androgens increase the number of bone cells. So far, gonadal androgens are the only androgenic steroids having been shown to exert direct proliferative action on

HBCs via androgen receptor-mediated mechanisms. To examine the possible mitogenic effects of the adrenal steroids DHEA and DHEAS and to compare the effects with the action of gonadal androgens, HBC populations were treated in parallel with adrenal androgens and DHT. DHEA and DHT stimulated HOC proliferation in a dose-dependent manner after treatment for 48 and 72 h (Table 1a). We did not find a reproducible stimulatory effect of DHEAS on HOC proliferation (data not shown). There was no qualitative difference between the effects of DHEA and DHT on HOC growth, but DHT, at doses at or greater than 0.1 nM, elicited a significantly more potent mitogenic response than DHEA after 48 h of treatment (Tables 1a and 2). The finding of a greater mitogenic effect of DHT compared with DHEA may be attributable to a greater affinity of DHT to the androgen receptor. To test this possibility, displacement binding experiments were performed as shown in Figure 1. DHT was more than 20-fold as potent in competing with the synthetic androgen R1881 for binding to androgen binding sites when compared with DHEA. There was no major difference between DHT and R1881 with regards to competing with labeled R1881 for binding to androgen binding sites of HOC. Therefore, the observation of a greater mitogenic effect of DHT (when compared with the mitogenic effect of DHEA on HOCs)

TABLE 2. EFFECTS OF 4-MA ON THE MITOGENIC ACTION OF DHT AND DHEA

	Concentration (nM)					
	0	0.01	0.1	1	10	100
DHT + 4-MA		150 ± 33*	183 ± 19	233 ± 27	225 ± 16	266 ± 27
	115 ± 29 (NS)	160 ± 86 (NS)	180 ± 40	190 ± 38	230 ± 20	290 ± 50
DHEA + 4-MA		100 ± 16 (NS)	135 ± 14	157 ± 16	214 ± 16	221 ± 14
	115 ± 29 (NS)	130 ± 15*	146 ± 15	161 ± 14	200 ± 17	246 ± 25

Human bone cells were treated with the indicated concentrations of DHT and DHEA in the absence and presence of 100 nM 4-MA for 48 h before the cells were counted with a hemocytometer (data presented as mean of % of control ± SD; control counts: 300 ± 80, $p < 0.01$; * $p < 0.05$; NS, not significant). DHT had a significantly greater effect on HBC proliferation than DHEA as determined by ANOVA.

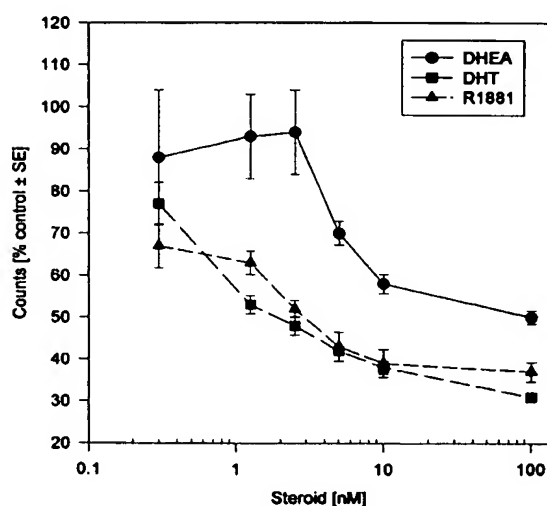


FIG. 1. Competing androgen binding in a human osteoblastic cell population. Binding was determined by using a displacement assay as described in Materials and Methods. Data presented are expressed as counts in mean percent of binding (\pm SE) relative to the binding in the absence of competing androgen (= control) for each competing steroid concentration from six separate experiments. The 50% displacement concentrations of the androgenic steroids were determined after logit-log transformation of the displacement curves: R1881, 3 nM; DHT, 3.3 nM; DHEA, 80 nM.

may be related to a greater affinity of DHT to the androgen receptor.

Previously, we showed that the positive effect of DHT on HBC proliferation can be inhibited by the androgen receptor antagonist hydroxyflutamide (OHFlu).⁽⁶⁾ To test whether the mitogenic effect of DHEA can also be inhibited by OHFlu, the dose response experiment was repeated in the presence of the androgen receptor antagonist OHFlu. OHFlu alone had no significant effect on HOC proliferation, but the addition of OHFlu to DHEA abolished the proliferative effect of DHEA on HOCs

(Table 1a). Therefore, the mitogenic actions of both DHT and DHEA on HBCs are mediated by the androgen receptor.

If DHT and DHEA exert their positive effects on HBC proliferation by an androgen receptor-mediated mechanism, it should be possible to demonstrate an additive effect of DHEA and DHT on HOC growth in the lower concentration range. At higher androgen doses, there should be no additive interaction because all available androgen receptors are occupied by either compound. The observed pattern of responses was consistent with the expected results. After incubation for 24 h, either with 1 nM DHT and cotreatment with increasing concentrations of DHEA or with 1 nM DHEA and cotreatment with increasing concentrations of DHT, it was found that DHEA and DHT were additive on HOC growth in the lower dose range but were not additive at the higher doses tested (Table 1b).

An alternative explanation for the direct mitogenic effect of DHEA is a possible metabolism of DHEA by HOCs into androstenedione, testosterone, and DHT, which then binds to the androgen receptor and elicits the observed proliferative effects. To investigate this possibility, we repeated the experiments testing the mitogenic effects of DHEA and DHT, but this time in the absence and presence of 4-MA, a 5- α -reductase inhibitor, which also has a potent inhibitory effect on 3 β -hydroxysteroid dehydrogenase (3 β -HSD). Inhibition of 3 β -HSD activity prevents the conversion of DHEA to androstenedione and testosterone, which could be further metabolized into aromatized compounds (e.g., 17 β -estradiol).⁽²³⁾ The presence of 4-MA did not affect the mitogenic actions of a wide dose range of either DHEA or DHT on HOC proliferation after 48 h of exposure (Table 2). This observation indicates that DHEA does not need to be metabolized by the actions of 3 β -HSD or 5- α -reductase to exert a direct proliferative effect on HOC proliferation.

Effects of adrenal and gonadal androgens on differentiated bone cell functions

A committed osteoblastic stem cell developing into a mature osteoblast expresses the ALP gene before the osteocalcin gene is turned on.⁽²⁴⁾ ALP stimulates mineral formation,⁽²⁵⁾ whereas the exact role of osteocalcin in the

TABLE 3. EFFECT OF INHIBITORY TGF- β ANTIBODIES ON THE STIMULATORY ACTION OF DHT AND DHEA ON SPECIFIC ALP ACTIVITY

	Concentration (nM)					
	0	0.01	0.1	1	10	100
DHT + TGF- β Ab		147 \pm 14	139 \pm 16	158 \pm 14	172 \pm 24	142 \pm 12
	102 \pm 7 (NS)	101 \pm 14 (NS)	106 \pm 18 (NS)	91 \pm 16 (NS)	94 \pm 16 (NS)	96 \pm 12 (NS)
DHEA + TGF- β Ab		125 \pm 9	140 \pm 28	148 \pm 16	158 \pm 20*	140 \pm 9
	102 \pm 7 (NS)	108 \pm 13 (NS)	116 \pm 15 (NS)	117 \pm 23 (NS)	108 \pm 17 (NS)	102 \pm 9 (NS)

Human bone cells were treated with DHT and DHEA in the absence and presence of 100 ng/ml TGF- β Ab for 72 h (data presented as mean of percent of control \pm SD; control: 58 \pm 4 U/g of protein, p < 0.01; * p < 0.05; NS, not significant). There is no significant difference between the stimulatory effects of both androgens on ALP (ANOVA).

TABLE 4. EFFECT OF 4-MA ON THE ACTION OF DHT AND DHEA ON THE NUMBER OF ALP POSITIVE STAINING HOC

	Concentration (nM)					
	0	0.01	0.1	1	10	100
DHT + 4-MA		98 \pm 6 (NS)	114 \pm 11*	130 \pm 9	139 \pm 7	179 \pm 14
	114 \pm 13 (NS)	120 \pm 6*	130 \pm 10	125 \pm 6	142 \pm 5	153 \pm 13
DHEA + 4-MA		96 \pm 7 (NS)	114 \pm 7	131 \pm 5	153 \pm 8	119 \pm 5
	114 \pm 13 (NS)	113 \pm 11 (NS)	125 \pm 8	129 \pm 13	141 \pm 9	121 \pm 4

Human bone cells were treated with the indicated concentrations of DHT and DHEA in the absence and presence of 100 nM 4-MA for 72 h (data presented as mean of percent of control \pm SD; control: 360 \pm 40, p < 0.01; * p < 0.05; NS, not significant). There is no consistent quantitative difference between the positive effects of DHEA and DHT on the number ALP⁺ HBC.

mineralization process is not known.⁽²⁴⁾ Because gonadal androgens (e.g., DHT) have been shown to stimulate ALP production, we examined the possibility that DHT and DHEA have different effects on ALP and osteocalcin expression and on the formation of mineral in vitro, respectively.

Previously, we reported that the positive effect of the gonadal androgen DHT on ALP appears to be mediated by transforming growth factor beta 2 (TGF- β 2)⁽²⁶⁾ which was also observed by Bodine et al.⁽¹⁴⁾ Therefore, we sought to confirm that (1) DHEA does also increase ALP activity in HOCs and (2) that the positive effects of both DHT and of the adrenal androgen DHEA on ALP are mediated by TGF- β 2. To this end, HOCs were treated with various doses of DHT and DHEA in the presence or absence of inhibitory TGF β -antibodies (TGF- β ab's). DHT and DHEA were found to have biphasic stimulatory effects on the ALP content of cells with maximal effective doses ranging between 1–10 nM (Table 3). There was no significant quantitative difference between the effects of DHT and DHEA on ALP activity. In the presence of inhibitory TGF- β ab, the stimulatory effects of DHT and DHEA on ALP were abolished. This observation is consistent with the hypothesis that the positive effects of DHT and DHEA on ALP are both mediated by increased expression of TGF- β .

The effect of DHEA on ALP activity was somewhat (although not significantly) smaller compared with the action of DHT on ALP. This could be due to an indirect effect of DHEA, which needs to be metabolized into another androgenic compound first. This androgenic compound

(e.g., testosterone) could then elicit a positive effect on ALP activity. To investigate this possibility, HOCs were treated with DHT and DHEA with and without 4-MA being present, and this time the number of ALP⁺ staining cells was determined. DHT and DHEA significantly increased the number of ALP⁺ HOCs, whereby we did not find a reproducible quantitative difference between the positive effects of DHT and DHEA on the number of ALP⁺ HOCs. The positive effects of DHT and DHEA on ALP⁺ cells were not changed by the presence of 4-MA (Table 4). Thus, similar to the mitogenic action, the stimulatory effect of DHT and DHEA on ALP is also direct and does not require 5- α -reductase or 3 β -HSD activity to convert the androgen into another active compound.

Increased osteocalcin secretion by bone cells occurs late in the osteoblastic differentiation process.⁽²⁴⁾ In vitro, increased osteocalcin secretion can be observed concomitant to the initiation of mineral formation. Osteocalcin secretion and mineral formation were measured after 3 and 7 days, respectively, to determine the effects of DHT and DHEA on the late osteoblastic differentiation processes. DHT and DHEA dose-dependently stimulated osteocalcin secretion with a maximum effect at 100 nM (Table 5). Both DHT and DHEA also significantly increased the formation of mineral in vitro. DHT was more potent than DHEA in this respect. Therefore, DHT and DHEA exhibit stimulatory effects on metabolic functions of HOCs (i.e., ALP production, osteocalcin secretion, and mineral formation) occurring at different stages of the osteoblastic differentiation process.

TABLE 5. EFFECTS OF DHT AND DHEA ON OSTEOCALCIN SECRETION AND MINERALIZATION IN CULTURE

(nM)	Osteocalcin*		Mineralization†	
	DHT	DHEA	DHT	DHEA
0.001	84 ± 18 (NS)	95 ± 21 (NS)	ND	ND
0.01	111 ± 20 (NS)	108 ± 14 (NS)	ND	ND
0.1	133 ± 12 [‡]	112 ± 8 [‡]	ND	ND
1	139 ± 14 [‡]	115 ± 6 [‡]	206 ± 27	116 ± 18 (NS)
10	145 ± 16	128 ± 11	233 ± 17	151 ± 21
100	151 ± 18	153 ± 20	236 ± 13	170 ± 29

* Human bone cells were treated with DHT and DHEA under serum-free conditions. After 72 h, secreted osteocalcin was measured in conditioned media and total cell protein in Triton X-100 cell extracts (data presented as mean of percent of control ± SD; control: 5 ± 1.3 mg/g protein; $p < 0.01$; [‡] $p < 0.05$; NS, not significant). There is no significant difference between the stimulatory effects of both androgens on osteocalcin secretion as analyzed by ANOVA. † After reaching confluency human bone cell populations were treated with DHT and DHEA for 7 days before 1 μ Ci/ml Ca^{45} was added for 24 h (data presented as mean of percent of control ± SD; Ca^{45} incorporated into mineral of control cultures: 9731 ± 1080 cpm; $p < 0.01$; NS, not significant; ND, not determined). DHT has a significantly greater effect on the stimulation of in vitro mineralization than DHEA (determined by ANOVA).

DISCUSSION

The role of adrenal androgens in regulating bone metabolism and their possible mechanism of action is not clear. Only recently have some of the effects of adrenal androgens on human bone cells been investigated in greater detail.⁽¹⁴⁾ We sought to extend our knowledge about the mechanism of action of adrenal androgens on human bone cells by comparing the effects of gonadal and adrenal androgens on human bone cell growth and osteoblastic functions. The results presented in this study demonstrate that DHT and the adrenal androgen DHEA have similar stimulatory actions on human bone cell proliferation and differentiated cell functions in vitro. Both DHT and DHEA increase human bone cell growth and stimulate osteoblastic functions occurring early (i.e., alkaline phosphatase expression) and late (i.e., osteocalcin secretion and mineralization) during osteoblastic differentiation.⁽²⁴⁾ Therefore, gonadal and adrenal androgens have similar effects on the regulation of bone cell metabolism. Quantitative differences between DHT and DHEA with regard to their mitogenic and differentiation stimulating effects may be related to differences in their binding affinities to the androgen receptor, which has previously been reported for the effects of DHT and testosterone.⁽³²⁾

Similar to the findings of Bodine et al.,⁽¹⁴⁾ we also observed similar effects of DHEA and DHT on ALP production and osteocalcin secretion. The finding of similar dose- and time-dependent effects of gonadal and adrenal androgens on HOC metabolism suggests a similar mechanism of action for both androgens. An additive effect of DHEA and DHT was observed on HOC proliferation in the lower concentration range. At higher androgen doses, there was no additive interaction, presumably because all available androgen receptors were occupied by either compound. If DHT and DHEA act through the same androgen receptor-mediated mechanism, the effects of

DHEA should also be blocked by the androgen receptor antagonist hydroxyflutamide, which has previously been demonstrated for DHT-induced effects on HOCs.⁽⁶⁾ The nonsteroidal androgen receptor antagonist hydroxyflutamide abolished the positive effect of DHEA on HOC proliferation, indicating that DHEA binds directly to the androgen receptor of HOC. Contrary to the present finding, Grover and Odell⁽²⁷⁾ did not observe DHEA binding to the cytosolic androgen receptor in rat prostate extracts. There are three possible explanations for this discrepancy. First, there may be a species-related difference in the binding characteristics of androgen receptors. The exchange of a single amino acid residue can have a major impact on the binding pattern of steroid receptors, as recently demonstrated for the murine glucocorticoid receptor.⁽²⁸⁾ Second, the protein extraction procedure of Grover and Odell may have altered the androgen receptor structure, for example by separating components of the androgen receptor heterotetrameric complex,⁽²⁹⁾ which may affect the binding characteristics of the androgen receptor protein.^(30,31) Third, DHEA may be converted into androstenedione, testosterone, and DHT, which could then bind to the androgen receptor and elicit the observed effects. In addition, these compounds may in turn be further metabolized by β -hydroxysteroid-dehydrogenase into aromatized bone-active compounds, such as 17 β -estradiol.⁽²³⁾ To examine the third possibility, HOCs were treated with DHEA in the presence of 4-MA, which blocks 5- α -reductase and β -hydroxysteroid dehydrogenase activity⁽²³⁾ and had no effect either on the stimulatory effects of DHEA on HOC proliferation or on the induced increase in the number of ALP positive staining bone cells. Consequently, the combined treatment with DHEA and 4-MA prevents a possible conversion of DHEA into testosterone, DHT, and estrogens. The presence of 4-MA did not affect the stimulatory effects of DHEA on HOC proliferation and on the increase in the number of ALP⁺ staining bone cells. This demonstrates

that DHEA acts directly on HOCs via binding to the androgen receptor and does not need to be metabolized first to testosterone and DHT.

The conclusion that both DHT and DHEA regulate HOC metabolism by the same mechanism is supported by the observation that both androgens induce TGF- β 2 secretion in HOC populations that stimulate ALP production in a paracrine fashion,⁽¹⁴⁾ and by our finding that inhibitory TGF- β antibodies abolished stimulatory effects of both DHT and DHEA on ALP production in HOC populations. This observation is consistent with the hypothesis that the positive effects of DHT and DHEA on ALP are mediated by TGF- β 2. In contrast to the findings of Bodine et al.,⁽¹⁴⁾ we observed equipotent effects for DHEA and DHT on HOC metabolism. Possibly this difference is due to differences in the homogeneities of the HOC populations studies. We used only male HOCs for our experiments, whereas Bodine et al. used pooled HOC populations of male and female origin. Whether male HOCs are more responsive than female HOCs to gonadal and adrenal androgens is currently under investigation. Certainly gonadal and adrenal androgens are potent regulators of growth and osteoblastic functions in males and females, and androgens are responsible for the sexual dimorphism observed in the skeleton.

Considering the relatively high serum concentrations of adrenal androgens compared with gonadal androgens and estrogens in both sexes, it is conceivable that adrenal androgens could play a more important role in the maintenance of bone mass than previously realized. Adrenal androgens may be particularly important in aging males and postmenopausal females when serum levels of testosterone and estrogens decline.

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Distinguishing Androgen Receptor Agonists and Antagonists: Distinct Mechanisms of Activation by Medroxyprogesterone Acetate and Dihydrotestosterone

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Natural and pharmacological androgen receptor (AR) ligands were tested for their ability to induce the AR NH₂-terminal and carboxyl-terminal (N/C) interaction in a two-hybrid protein assay to determine whether N/C complex formation distinguishes *in vivo* AR agonists from antagonists. High-affinity agonists such as dihydrotestosterone, mibolerone, testosterone, and methyltrienolone at concentrations between 0.1 and 1 nM induce the N/C interaction more than 40-fold. The lower affinity anabolic steroids, oxandrolone and fluoxymesterone, require concentrations of 10–100 nM for up to 23-fold induction of the N/C interaction. However no N/C interaction was detected in the presence of the antagonists, hydroxyflutamide, cyproterone acetate, or RU56187, at concentrations up to 1 μ M, or with 1 μ M estradiol, progesterone, or medroxyprogesterone acetate; each of these steroids at 1–500 nM inhibited the dihydrotestosterone-induced N/C interaction, with medroxyprogesterone acetate being the most effective. In transient and stable cotransfection assays using the mouse mammary tumor virus reporter vector, all ligands displayed concentration-dependent AR agonist activity that paralleled induction of the N/C interaction, with antagonists and weaker agonists failing to induce the N/C interaction. AR dimerization and DNA binding in mobility shift assays and AR stabilization reflected, but were not dependent on, the N/C interaction. The results indicate that

the N/C interaction facilitates agonist potency at low physiological ligand concentrations as detected in transcription, dimerization/DNA binding, and stabilization assays. However the N/C interaction is not required for agonist activity at sufficiently high ligand concentrations, nor does its inhibition imply antagonist activity. (Molecular Endocrinology 13: 440–454, 1999)

INTRODUCTION

Androgen receptor (AR) function is required for male sex development in the fetus, virilization at puberty, and maintenance of reproductive function in the adult. Interruption of these processes by pharmacological androgen antagonists or environmental endocrine disruptors can cause incomplete masculinization of the fetus or possibly reduced male fertility later in life (1, 2). Overstimulation of the prostate by androgen agonists may promote prostate cancer (3). Experimental approaches to identify and distinguish AR agonists from antagonists would aid in the classification of environmental and pharmaceutical chemicals since ligand-binding affinity alone does not necessarily reflect biological potency, and transient transcriptional assays can be hampered by the complexity of the systems.

Previous studies from this laboratory identified an AR NH₂-terminal and carboxyl-terminal (N/C) interaction that requires high-affinity androgen binding (4). The androgen-induced N/C interaction is inhibited by the androgen antagonist hydroxyflutamide. These results raised the possibility that an N/C interaction is

required for AR agonist activity and that its interruption is a prerequisite for antagonist activity. Similar studies with the estrogen receptor revealed a ligand-dependent N/C interaction that predicted parallel dimerization (5). Recent studies on AR suggest that its N/C interaction is intermolecular and results in the formation of an antiparallel homodimer (6). A feature common to both models is the requirement for high-affinity agonist binding to promote the N/C interaction. In the present report we tested the requirement for the AR N/C interaction in relation to AR dimerization, DNA binding, and transcriptional activity in transient and stable cotransfection assays to distinguish the activities of several natural and pharmaceutical agonists and antagonists. The results suggest that at higher concentrations, certain weak AR agonists such as medroxyprogesterone acetate (MPA) activate AR through a mechanism that does not involve the N/C interaction, although potent agonists capable of AR activation at low ligand concentrations induce the N/C interaction. Furthermore, inhibition of the N/C interaction does not necessarily reflect the activity of an antagonist.

RESULTS

Ligand Binding Affinities

Binding affinities of ligands listed in Table 1 are indicated as apparent equilibrium binding constant (K_d) determined by Scatchard analysis as previously reported, inhibition constant K_i of endogenous AR in rat

prostate extracts, or the concentration required for 50% inhibition of [3 H]methyltrienolone (R1881) binding to recombinant AR in transfected COS cells (Table 1). Relative competitive binding affinities for [3 H]R1881 in transfected COS cells were mibolone \sim R1881 $>$ RU56187 $>$ dihydrotestosterone (DHT) \sim MPA $>$ progesterone $>$ estradiol (E_2) $>$ cyproterone acetate $>$ testosterone \sim oxandrolone \sim fluoxymesterone $>$ hydroxyflutamide. Chemical structures of several of the ligands are shown in Fig. 1. Binding of DHT and testosterone was weaker in the COS cell assay when compared with apparent equilibrium binding affinities in tissue cytosols (Table 1) likely resulting from partial metabolism during the 2-h 37°C incubation. Analysis of COS cells transfected with the parent plasmid lacking the AR sequence showed binding only of [3 H]progesterone. Lack of binding of [3 H]R1881 and [3 H]mibolone in the absence of AR expression suggested that this endogenous binding activity was not due to the progesterone receptor (7–10). Inhibition constants for the anabolic steroids oxandrolone (K_i 62 nM) and fluoxymesterone (K_i 44 nM) were less than that of hydroxyflutamide (K_i 175 nM), but about 100 times greater than the equilibrium binding constant (K_d) for high affinity agonists such as DHT. Competitive binding by MPA and RU56187 were similar to DHT, although the reported K_d values for MPA were slightly greater than that for DHT (Table 1).

N/C Interaction

Induction of AR N/C complex formation was determined in a two-hybrid protein assay using Chinese

Table 1. Summary of Human AR Ligand Binding Affinities, Ligand-Induced N/C Interaction, and Transcriptional Activation and Inhibition

	K_d (nM)	K_i (nM)	Competitive Binding (nM)	Agonist		Antagonist	
				N/C	MMTV	N/C	MMTV
				(nM)	(nM)	(nM)	(nM)
DHT	0.2–0.5		100	0.1	0.001	— ^a	—
Mibolone	0.53		12	0.1	0.001	—	—
Testosterone	0.2–0.5		220	1	0.01	—	—
R1881	0.6		15	1	0.001	—	—
Oxandrolone		62	260	10	1	—	—
Fluoxymesterone		44	300	100	1	—	—
MPA	1.7–3.6		75	—	0.1	1	—
RU56187	0.39		35	—	10	10	100
Estradiol		110	120	—	10	50	500
Progesterone			62	—	100	50	500
Cyproterone acetate			170	—	100	50	100
Hydroxyflutamide		175	460	—	10,000	50	100

The apparent equilibrium binding constant (K_d , nM) is indicated for DHT (62), mibolone (63), testosterone (62), R1881 (64), MPA (28, 42), and RU56187 (14). The K_i (nM) is indicated for E_2 (1), oxandrolone, fluoxymesterone, cyproterone acetate, and hydroxyflutamide. Approximate unlabeled ligand concentrations (nM) that cause 50% competitive inhibition of 5 nM [3 H]R1881 binding to recombinant human AR expressed in COS cells were determined as described in *Materials and Methods*. Ligand concentrations (nM) that induce at least 10-fold agonist activity were determined in the two-hybrid N/C interaction assay in CHO cells (N/C) and transcriptional activation of MMTV-luciferase in CV1 cells (MMTV). Ligand concentrations (nM) for 50% inhibition (antagonist activity) were determined in the two-hybrid N/C interaction assay in CHO cells in the presence of 1 nM DHT (N/C) or with the MMTV-luciferase reporter in CV1 cells in the presence of 0.1 nM DHT (MMTV).

^a Dashes indicate not detectable.

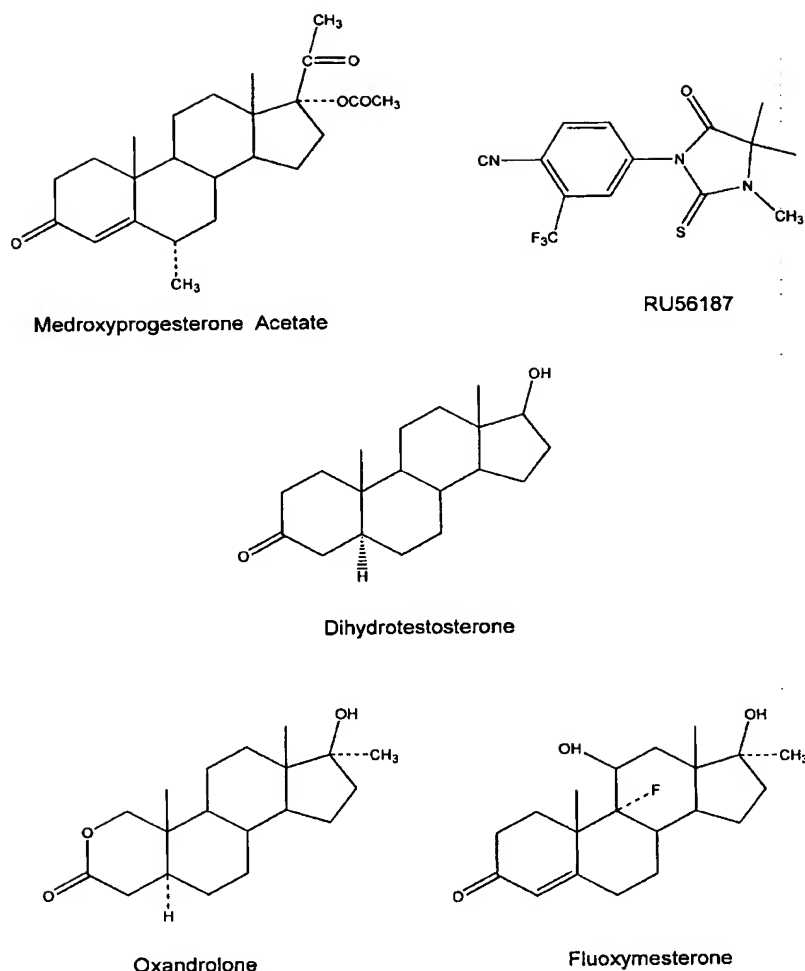


Fig. 1. Chemical Structures for MPA, RU56187, DHT, Oxandrolone, and Fluoxymesterone

hamster ovary (CHO) cells as previously described (4, 6). Relative ligand potency between 0.1 nM and 1 μ M was DHT ~ mibolerone > testosterone ~ R1881 > oxandrolone > fluoxymesterone (Fig. 2). No N/C interaction was detected with MPA, RU56187, E_2 , progesterone, hydroxyflutamide, or cyproterone acetate up to concentrations of 1 μ M. Induction of the N/C interaction did not correlate with relative binding affinities (Fig. 2 and Table 1). The relatively high-affinity ligands, MPA and RU56187, failed to promote the N/C interaction, whereas the lower affinity anabolic steroids, oxandrolone and fluoxymesterone, induced the N/C interaction. The inability of MPA to induce the N/C interaction was not limited to CHO cells, as it was also ineffective in monkey kidney CV1 or COS cells where 5-fold induction of the N/C interaction was observed with 1 nM DHT (data not shown).

Inhibition of the DHT-induced N/C interaction by hydroxyflutamide reported previously (4) raised the

possibility that this inhibition may be necessary for and indicative of androgen antagonist activity. Because MPA is a weak AR agonist *in vivo* (11–13), it was surprising that MPA at concentrations as low as 10 nM blocked the DHT-induced N/C interaction and was about 50 times more potent than hydroxyflutamide as an inhibitor (Fig. 3 and Table 1). RU56187 was a slightly less potent inhibitor of the N/C interaction than MPA, exhibited a high AR equilibrium binding affinity, and is reported to have antagonist activity *in vivo* (14, 15). Ligands with less inhibitory activity than MPA or RU56187 at concentrations between 50 and 500 nM were hydroxyflutamide, cyproterone acetate, E_2 , and progesterone (Fig. 3 and Table 1). The anabolic steroids, oxandrolone and fluoxymesterone, and the potent androgen agonists, DHT, mibolerone, testosterone, and R1881, showed little or no inhibition of the DHT-induced N/C interaction.

To investigate the possibility that MPA induces an AR carboxyl-terminal/carboxyl-terminal (C/C) interac-

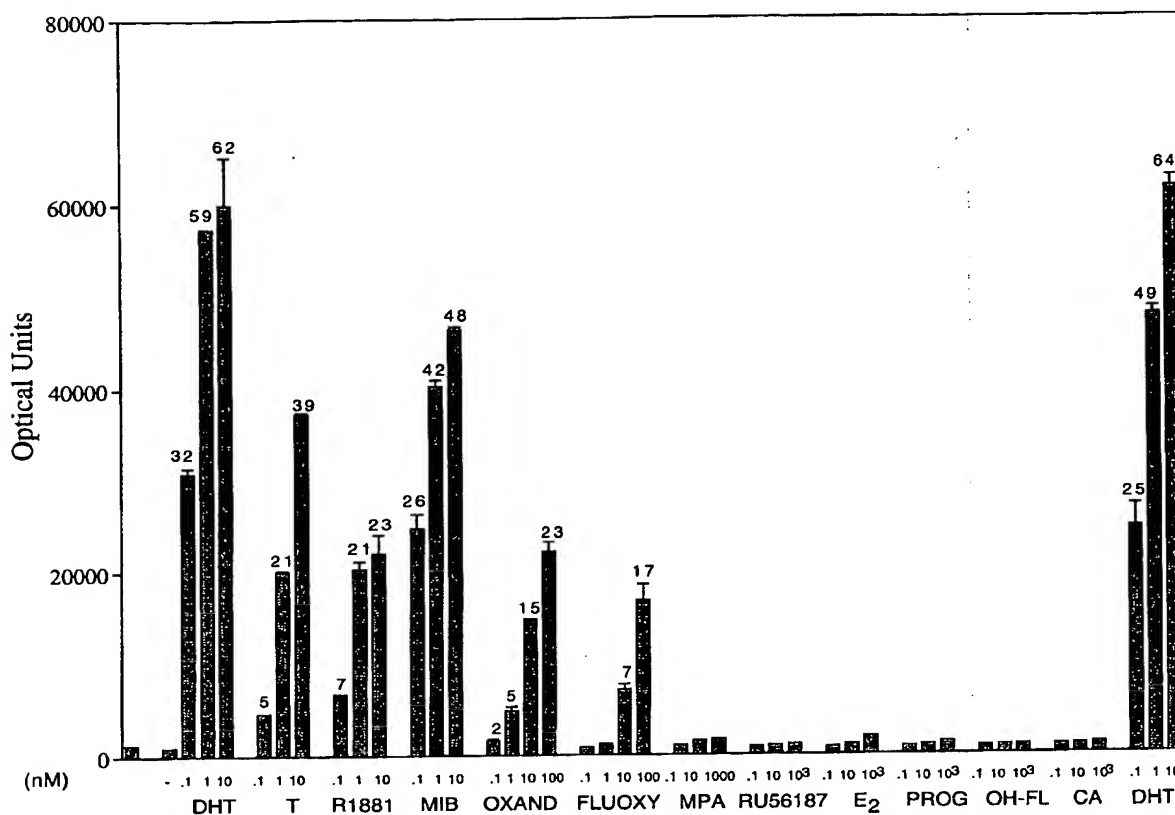


Fig. 2. Ligand Dependence of the AR N/C Interaction Determined in a Two-Hybrid Protein Assay in CHO Cells

CHO cells were transfected with GALD-H and VPAR1-660 human AR fusion protein expression vectors and the G5E1b-luciferase reporter vector as described in *Materials and Methods*. Cells were incubated with increasing concentrations of DHT, T (0.1–100 nM), methyltrienolone (R1881), mibolerone (MIB) (0.1–10 nM), oxandrolone (OXAND), fluoxymesterone (FLUOXY) (0.1–100 nM), MPA, RU56187, E₂, progesterone (PROG), hydroxyflutamide (OH-FL), and cyproterone acetate (CA) (0.1–1000 nM) as indicated. Shown are the optical luciferase units, and above the bars, the fold induction relative to the activity determined in the absence of ligand. The data are representative of at least three independent experiments.

tion, we tested GALD-H with VPD-H in the two-hybrid assay. VPD-H contains the VP16 transactivation domain linked as a fusion protein to the AR hinge and steroid-binding domain amino acid residues 624–919 and was used previously to demonstrate lack of a C/C interaction induced by DHT (4). Neither MPA nor DHT induced a C/C interaction in this assay more than 2-fold (results not shown).

Transcriptional Activation

Agonist and antagonist activities were determined in CV1 cells transiently transfected with a mouse mammary tumor virus (MMTV)-luciferase reporter and full-length human AR expression vectors. Ligands with more than 10-fold agonist activity at 0.001 nM were DHT, mibolerone, and R1881 (Fig. 4 and Table 1). Similar induction was achieved by 0.01 nM testosterone, 0.1 nM MPA, and 1 nM oxandrolone or fluoxymesterone. Cyproterone acetate, progesterone, E₂, and

RU56187 induced luciferase activity at concentrations between 10 and 100 nM, but transcriptional activity remained low at 100 nM hydroxyflutamide, the latter requiring concentrations of 1–10 μ M for agonist activity in this assay (16). Agonist potency, therefore, tended to parallel the ligand-induced N/C interaction. Lack of an N/C interaction induced by MPA is associated with 100-fold higher MPA concentrations necessary for transcriptional activity compared with DHT.

When transcriptional activity was tested in the same cells (CHO cells) used for the N/C two-hybrid assay but using the MMTV-luciferase reporter, 10- to 100-fold higher MPA concentrations were also required relative to DHT. In CV1 cells transfected with the MMTV-luciferase reporter and the pCMV5 parent plasmid lacking AR sequence, there was no induction of luciferase activity by MPA or any other ligand tested, ruling out the possibility that MPA activity was mediated through an endogenous receptor or altered lucif-

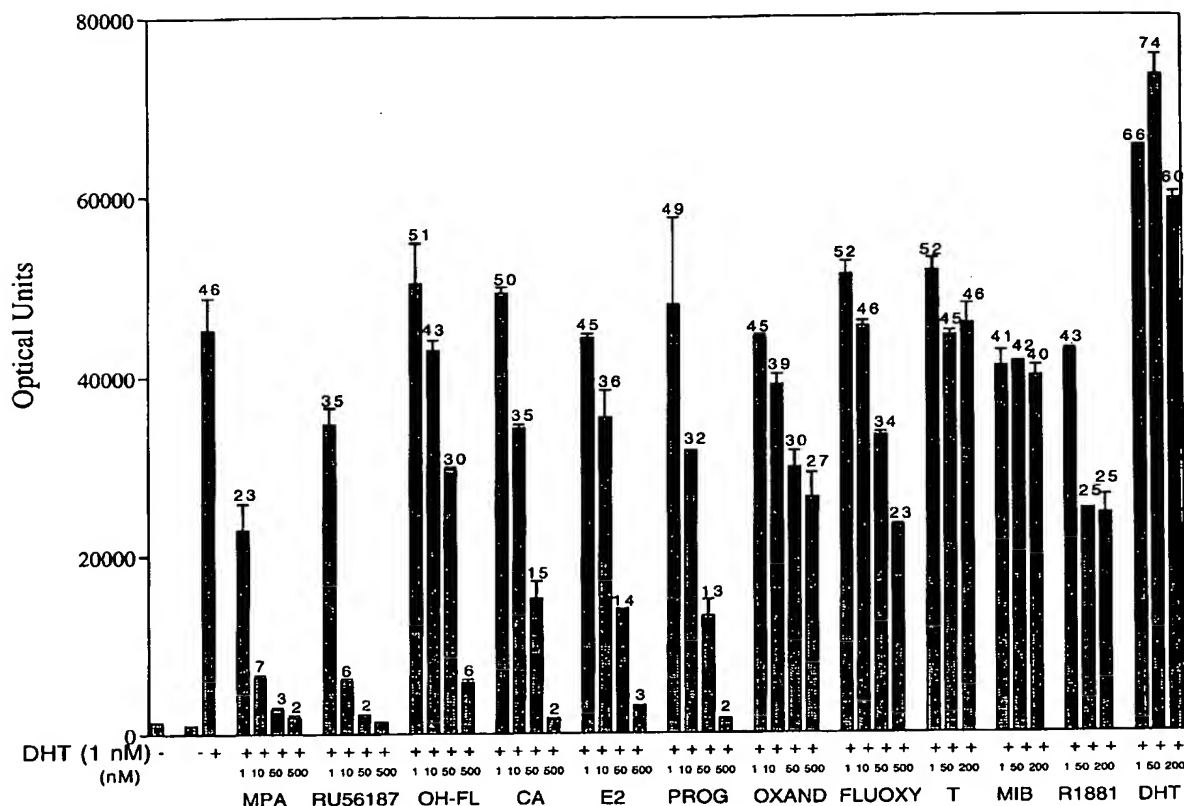


Fig. 3. Inhibition of the DHT-Induced AR N/C Interaction Determined in the Two-Hybrid Protein Interaction Assay in CHO Cells
CHO cells were transfected with the AR fusion expression vectors and the G5E1b-luciferase reporter vector as described in *Materials and Methods* and incubated with and without 1 nM DHT or in the presence of 1 nM DHT with increasing concentrations of the indicated ligands (abbreviations as in Fig. 2 legend). Optical luciferase units are shown with the fold induction relative to the activity determined in the absence of ligand indicated above the bars. The data are representative of at least three independent experiments.

erase expression by a nonreceptor mechanism. A luciferase reporter vector with two copies of the MMTV glucocorticoid response element separated by a 29-bp linker derived from pMTV29VTM (17) and cloned into pT81Luc (18) also had greater agonist activity with DHT relative to MPA (data not shown).

Antagonist activity of the ligands was tested in CV1 cells by coinubation with 0.1 nM DHT. Hydroxyflutamide was the most effective antagonist with about 50% inhibition at 100 nM (Fig. 5). Cyproterone acetate was slightly less effective, and RU56187 had some inhibitory activity but decreased in effectiveness at higher concentrations. Antagonist activity was also observed with increasing concentrations of progesterone and E₂, but none was observed with MPA (Fig. 5) or with the high-affinity agonists or the anabolic steroids (results not shown). Thus, except for MPA, at least partial inhibition of DHT-induced transcriptional activity correlated with inhibition of the DHT-induced N/C interaction.

The inability of MPA to induce the N/C interaction but have agonist rather than antagonist activity in transient transcription assays and *in vivo* (11–13, 19) prompted us to investigate whether two AR mutants that cause severe androgen insensitivity might be selectively activated by MPA in transient cotransfection assays. The mutants, V889M and R752Q, each retain high-affinity equilibrium binding of [³H]R1881 (20–22) but are defective in the N/C interaction (6). V889M had a similar blunted response to both DHT and MPA in the MMTV-luciferase reporter assay in CV1 cells, whereas R752Q required about 10-fold higher concentrations of MPA (10 nM) relative to DHT to induce transcription (data not shown). Thus, neither mutant defective in the N/C interaction was efficiently activated by MPA or DHT, suggesting that these regions of the ligand-binding domain are important in AR activation by both steroids.

The weak *in vivo* AR agonist activity reported for MPA (11–13, 19) is reflected in MMTV-luciferase as-

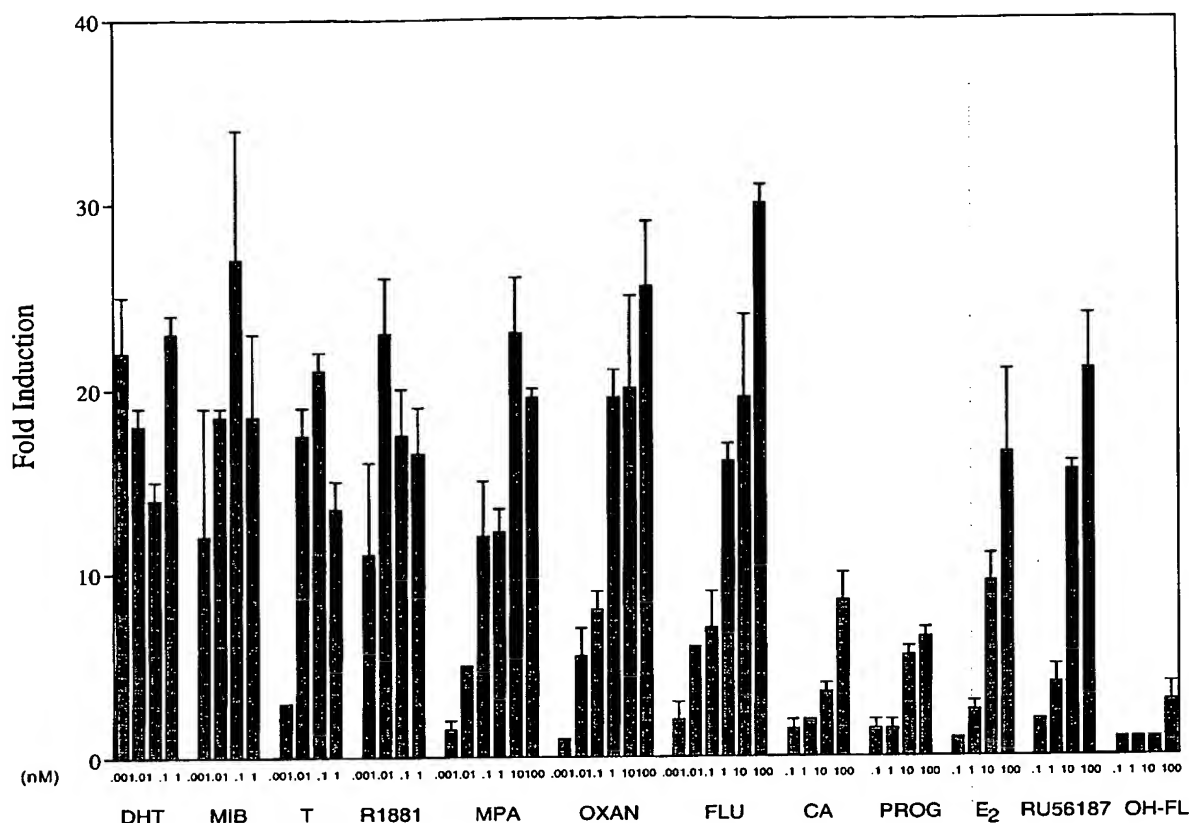


Fig. 4. Ligand-Dependent AR-Mediated Transcriptional Activation of the MMTV-Luciferase Reporter Vector in CV1 Cells

CV1 cells were transfected with 0.1 μ g pCMVhAR full-length human AR expression vector and 5 μ g MMTV-luciferase reporter vector using calcium phosphate as described in *Materials and Methods*. Transfected cells were incubated with increasing concentrations of the indicated ligands (abbreviations as in Fig. 2 legend). Shown is the fold induction of luciferase activity relative to the activity determined in the absence of ligand. The data are representative of at least three independent experiments.

says by the requirement for higher MPA concentrations relative to DHT for reporter gene activation. Nevertheless, the agonist activity of MPA was surprising considering that MPA inhibits the N/C interaction better than most antagonists. We therefore tested a CHO cell line in which the MMTV-luciferase reporter and human AR expression vectors were stably integrated in the genome using pcDNA3.1/Zeo vector with the zeocin gene and the human AR-coding sequence (K. Bobseine and W. R. Kelce, unpublished data). Cell lines such as this were used previously to distinguish agonist activities not detected by transient transfection (24). DHT at 0.1 nM stimulated luciferase activity 2-fold while MPA required a 10-fold higher concentration for similar induction (data not shown). A greater overall response to MPA (10-fold) compared with DHT (6-fold) likely resulted from MPA activation of endogenous glucocorticoid receptor since coinubation with 500 nM hydroxyflutamide inhibited MPA-activated gene transcription about 50% and DHT activity by 95%, but had no effect on induction by dexamethasone (data not shown). The AR-mediated MPA re-

sponse was therefore similar to that of DHT, but required higher steroid concentrations as observed in the transient assays. The chromatin arrangement of the reporter gene seemed to have little effect on the relative concentration-dependent gene activation by DHT and MPA.

Dimerization and DNA Binding

Because binding of baculovirus expressed full-length AR to androgen response element DNA requires exposure of Sf9 cells to androgen and is inhibited by coinubation with the antagonist hydroxyflutamide (25), we tested the activity of these ligands to promote AR DNA binding *in vitro*. DNA binding of full-length AR was observed with 50 nM DHT, mibolerone, MPA, oxandrolone, fluoxymesterone (Fig. 6A), or 50 nM R1881 or testosterone (Fig. 6B). Concentrations of DHT or R1881 less than 50 nM reduced AR DNA binding probably due to insufficient saturation of baculovirus-expressed AR (data not shown). AR DNA binding was

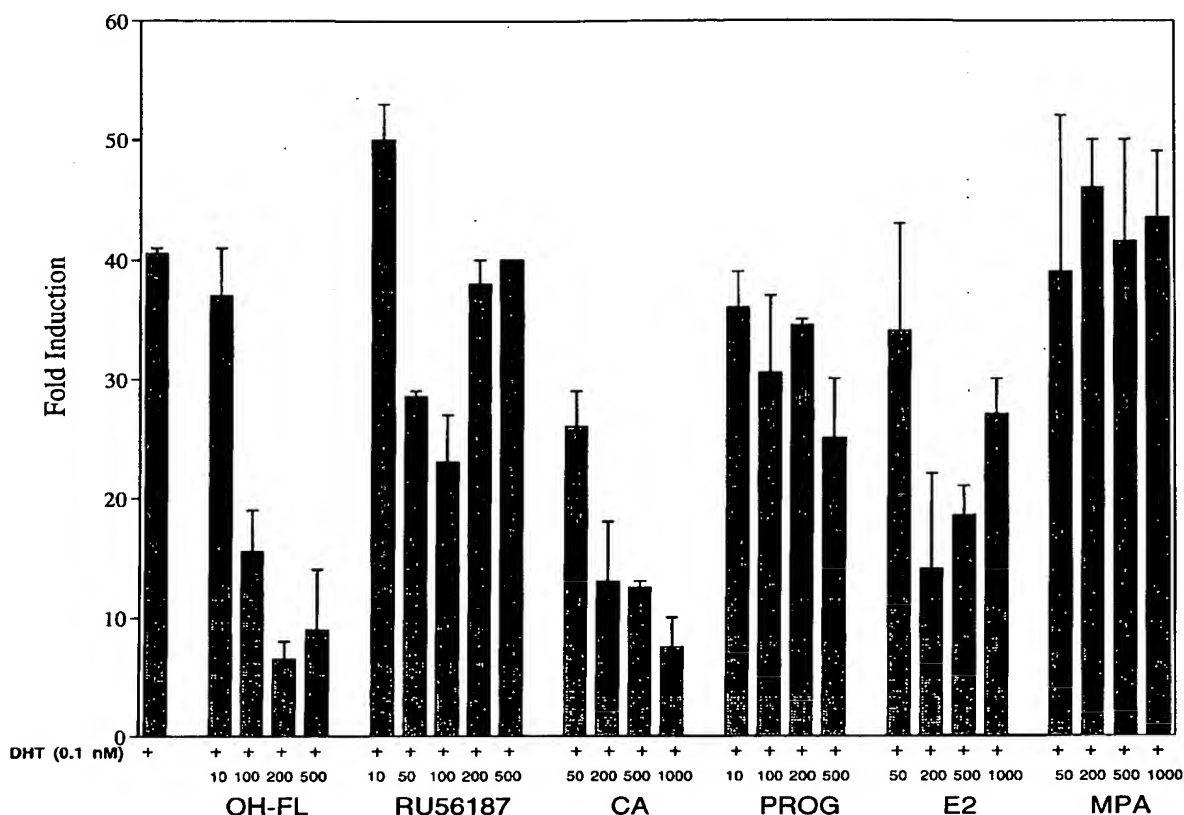


Fig. 5. AR Antagonist Activity in CV1 Cells

CV1 cells were transfected with 0.1 μ g pCMVhAR and 5 μ g MMTV-luciferase reporter vector as described in *Materials and Methods* and incubated in the presence and absence of 0.1 nM DHT or in the presence of 0.1 nM DHT with increasing concentrations of the indicated ligands (abbreviations as in Fig. 2 legend). Shown is the fold induction relative to activity determined in the absence of DHT. The data are representative of at least three experiments.

observed at 1 μ M RU56187 or hydroxyflutamide but was barely detectable with 1 μ M progesterone, E₂, or cyproterone acetate (Fig. 6).

Dimerization and DNA binding of baculovirus-expressed AR NH₂- and carboxyl-terminal fragments that contain the DNA-binding domain were also shown previously to distinguish androgen agonists and antagonists (25). While the NH₂-terminal and DNA-binding domain fragment AR1-660 (not shown) and the DNA-binding and carboxyl-terminal fragment AR507-919 (Fig. 7, lanes C) each homodimerize and bind DNA independently of hormone, agonists are required for dimerization and DNA binding of the N/C complex and an antagonist such as hydroxyflutamide inhibits this DHT-induced DNA binding (25, 26). Since both fragments contain the DNA-binding domain, dimerization could be mediated by the DNA-binding domain and/or by the N/C interaction. Results of this assay (25) and others (22) nevertheless predicted the AR N/C interaction, which was later confirmed in the two-hybrid interaction assay (4).

Dimerization and DNA binding of the AR fragments were observed at 50 nM DHT, mibolerone, testosterone, and R1881 (Fig. 7A, C+N) but required 0.5–1 μ M oxandrolone or fluoxymesterone for similar activity. MPA-induced dimerization and DNA binding of the two AR fragments were somewhat less efficient than DHT but similar to the anabolic steroids (Fig. 7, A and B). Only slightly weaker DNA binding of the N/C hybrids was detected using cyproterone acetate and E₂, whereas 1 μ M RU56187 or 1 μ M progesterone was required for DNA binding, and essentially no DNA binding was detected with 1 μ M hydroxyflutamide (Fig. 7 and Table 2). Binding of the homodimer C fragment alone was most effective with mibolerone, testosterone, and R1881; it was not detected with 50 nM DHT, required 500 nM MPA, and was weak to undetectable with the anabolic steroids and other ligands (Fig. 7, lanes C). Similar high-level AR expression was observed by immunoblot analysis of full-length AR or the AR507-919 fragment after the different hormone treatments and expression levels were independent of the

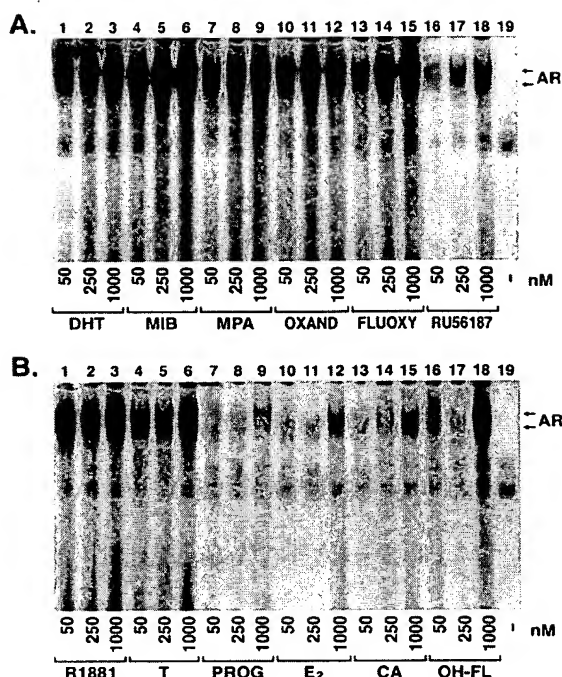


Fig. 6. Ligand-Dependent DNA Binding of Full-Length Human AR Expressed in Baculovirus

Sf9 cells expressing human AR were incubated with increasing concentrations of ligands as described in *Materials and Methods*. Cells were extracted in high-salt buffer, dialyzed to reduce the salt content, and incubated with 32 P-labeled androgen response element DNA as described in *Materials and Methods*. Shown is a reproduction of films in which the free 32 P-labeled oligo bands were removed from the bottom. In panels A and B, 50, 250, and 1000 nM of the indicated ligands were added in lanes 1–18 (abbreviations as in Fig. 2 legend). Lane 19 contains extracts from cells left untreated with recombinant virus or ligand. The AR- 32 P-oligo complex typically migrates as a double band (indicated with arrows) with the upper more slowly migrating band being predominant. In the absence of ligand the AR- 32 P-oligo complex is not detected (25).

extent of AR dimerization and DNA binding (data not shown).

Thus, MPA, cyproterone acetate, E_2 , and progesterone at concentrations between 50 nM and 1 μ M induce dimerization and DNA binding of the NH_2 - and carboxyl-terminal AR fragments, where both fragments contain the AR DNA-binding domain (Fig. 7B). Yet none of these ligands, except for MPA, promote DNA binding of full-length AR and none induce the N/C two-hybrid interaction (Fig. 7A). The effectiveness of these ligands to induce dimerization and DNA binding of the AR NH_2 - and carboxyl-terminal fragments in mobility shift assays is confounded by the presence of the dimerization region in the DNA-binding domain. We therefore attempted to address the contribution of the AR DNA-binding domain in the two-hybrid assay

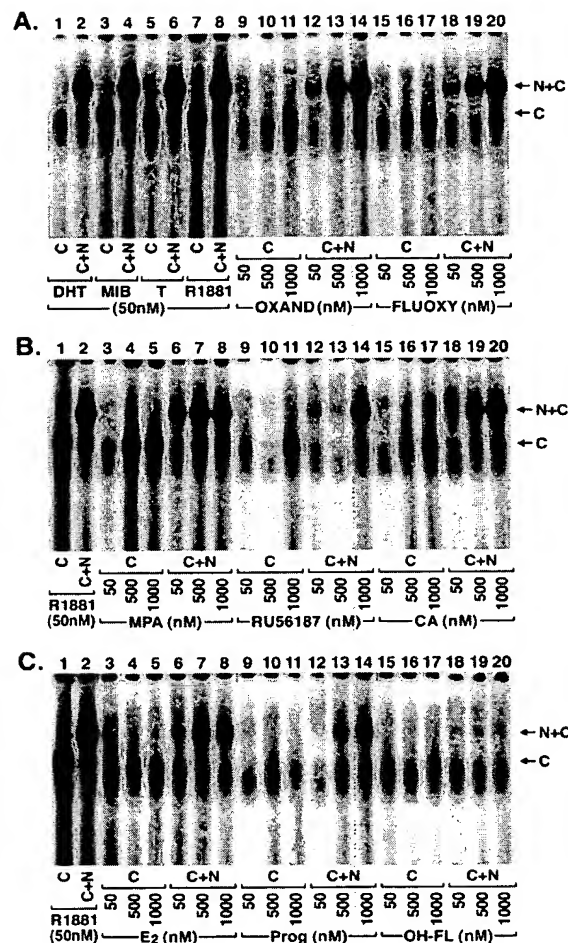


Fig. 7. Ligand Dependence of the AR NH_2 -Terminal and Carboxyl-Terminal Fragment Dimerization and DNA Binding in DNA Mobility Shift Assays

Human AR NH_2 -terminal DNA-binding domain fragment AR1–660 (N) and DNA ligand-binding domain-carboxyl terminal fragment AR507–919 (C) were expressed separately in Sf9 cells. Cells expressing C were incubated at the indicated ligand concentrations. After high-salt extraction and dialysis to lower the salt concentration, 20 μ g total protein of N or C or 10 μ g each of the N- and C-terminal fragments were combined and analyzed in DNA mobility shift assays as described in *Materials and Methods*. Cells expressing C were incubated with 50 nM DHT, mibolerone (MIB), testosterone (T), and R1881 (shown in panel A) along with 50, 500, and 1000 nM oxandrolone (OXAND) and fluoxymesterone (FLUOXY). R1881 (50 nM) is repeated in panels B and C as a control along with 50, 500, and 1000 nM MPA, RU56187, and cyproterone acetate (CA) (panel B), and the same concentrations of E_2 , progesterone (Prog), and hydroxyflutamide (OH-FL) (panel C). Shown are reproductions of films where the free 32 P-oligo band was cut from the bottom. Migration of the N + C and C complexes is indicated by arrows.

Table 2. Summary of AR Stabilization and Half-Times of [³H]Ligand Dissociation, AR DNA Binding, and *in Vivo* Activity of ligands

	Stability (nM)	[³ H]Ligand Dissociation Rate (h)	DNA Binding (nM)	<i>In Vivo</i> Activity
DHT	1	3.4	50	Agonist
Mibolerone	1	3.5	50	Agonist
Testosterone	5	1.2	50	Agonist
R1881	1	3.7	50	
Oxandrolone	10		500	Agonist
Fluoxymesterone	10		500	Agonist
MPA	100		50	Agonist
RU56187	— ^a	0.08	1000	Antagonist
E ₂	—	0.67	1000	
Progesterone	1000		1000	
Cyproterone acetate	1000		1000	Antagonist
Hydroxyflutamide	—		—	Antagonist

Ligand-dependent AR degradation was determined at 37 °C in COS cells using [³⁵S]methionine-labeled AR as described in Fig. 8. Indicated are the approximate ligand concentrations required to reduce AR degradation to approximately $t_{1/2} \geq 5$ h at 37 °C. Half-times of [³H]ligand dissociation (h) were determined at 37 °C in COS cells for DHT, testosterone, and R1881 (22), and for RU56187 and E₂ as described in Fig. 9. Ligand-dependent AR DNA binding was determined in mobility shift assays as described in Fig. 6. Shown are the ligand concentrations that effected DNA binding of baculovirus-expressed full-length human AR. *In vivo* agonist and antagonist activities were as previously reported for oxandrolone (45, 46), fluoxymesterone (46), MPA (11–13, 19), RU56187 (14, 15), cyproterone acetate (65), and hydroxyflutamide (66, 67).

^a Dashes indicate not detectable up to a concentration of 1 μ M.

by testing the interaction of a fusion protein comprised of the GAL4 DNA-binding domain and AR DNA and ligand-binding domains (GAL-AR507–919) with VPAR1–660 or VPAR. However, lack of an interaction in this and additional experiments using GAL-AR (GAL4 DNA-binding domain-full-length AR fusion protein) suggests that the presence of two DNA-binding domains in a fusion protein (*i.e.* from GAL4 and AR) interferes with hybrid formation.

AR Stabilization

One property that has distinguished androgen agonists from antagonists is their ability to stabilize AR against degradation (27). It was therefore of interest to determine the concentration dependence of AR stabilization by MPA, RU56187, and the anabolic steroids. Transfected COS cells were incubated with [³⁵S] methionine/cysteine and increasing concentrations of ligands, followed by chase periods with unlabeled methionine for 2–7 h as previously described (27). The results shown for several ligands in Fig. 8 and summarized in Table 2 indicate that more than 100 nM MPA was required to increase the AR degradation half-time to almost 5 h at 37 °C (Fig. 8C). A similar degree of AR stabilization was achieved by 1 nM DHT, mibolerone, or R1881, 5 nM testosterone (Table 2), or 10 nM fluoxymesterone or oxandrolone (Fig. 8, B and D). Cyproterone acetate and progesterone stabilized AR only at 1 μ M (Table 2), and almost no AR stabilization was observed with 1 μ M RU56187 (Fig. 8A), hydroxyflutamide, or E₂ (Table 2). The results tend to parallel the MMTV-luciferase and DNA-binding activities in that ligands that efficiently stabilize AR are more effective

agonists. It is noteworthy that the lower affinity anabolic steroids, oxandrolone and fluoxymesterone, promote the N/C interaction and stabilize AR at concentrations of 5–10 nM, concentrations only slightly higher than those required for the high-affinity agonists, DHT, mibolerone, R1881, and testosterone. However, higher concentrations of the anabolic steroids were required for DNA binding of the AR fragments, perhaps reflecting the lower AR binding affinity for these ligands.

Ligand Dissociation Rates

Because only few ligands could be obtained in ³H-labeled form, dissociation rates could not be determined for MPA, oxandrolone, fluoxymesterone, cyproterone acetate, or hydroxyflutamide. Nevertheless, we determined that [³H]RU56187 dissociates rapidly from AR with a $t_{1/2}$ of 5 min at 37 °C (Fig. 9) compared with $t_{1/2}$ of 2.5–3.5 h for [³H]R1881 (Fig. 9), [³H]DHT, and [³H]mibolerone (Table 2). A rapid dissociation rate for [³H]estradiol ($t_{1/2}$ 0.67 h) (Table 2) was also observed. The results raise the possibility that rapid ligand dissociation is associated with the lack of an N/C interaction and with *in vivo* antagonist activity.

DISCUSSION

Failure of MPA to induce the N/C interaction suggests that during AR dimerization, DNA binding, and gene activation, MPA activates AR by a mechanism different from other agonists. With the other ligands tested, agonist activity correlated with induction of the N/C

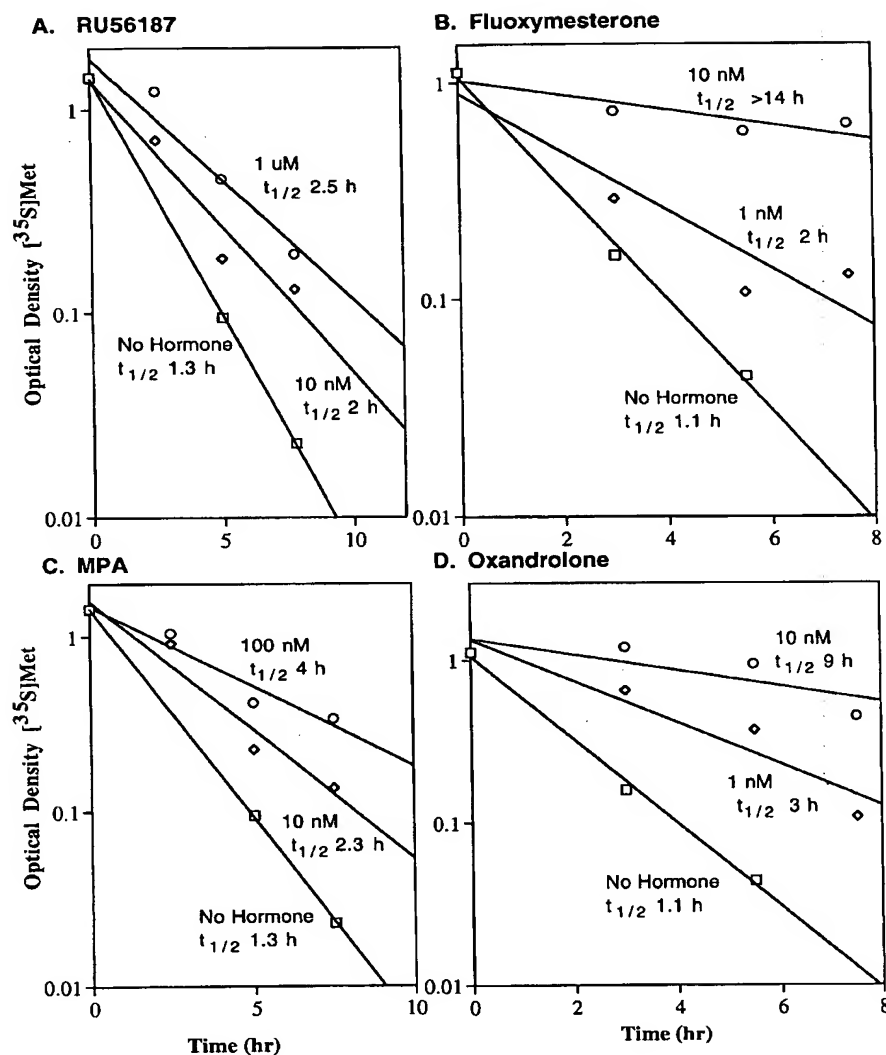


Fig. 8. Ligand-Dependent AR Stabilization

Full-length AR was expressed in COS cells from pCMVhAR as described in *Materials and Methods* and incubated in the presence of [35 S]methionine/cysteine for 30 min at 37 C followed by incubation with unlabeled methionine medium for increasing times in the presence of the indicated concentrations of ligand. Samples were extracted in RIPA buffer and AR was immunoprecipitated and analyzed on SDS polyacrylamide gels as previously described (22). Scanning the exposed films resulted in optical density readings of the AR bands, which migrated at approximately 114 kDa. Shown are the optical density measurements on a semilog scale. Approximate half-times of AR degradation at 37 C at the indicated ligand concentrations are shown on the figures for RU56187 (panel A), fluoxymesterone (panel B), MPA (panel C), and oxandrolone (panel D).

interaction and antagonist activity with its inhibition. MPA is a weak androgen *in vivo* (see below) which perhaps relates to its inability to induce the N/C interaction. Induction of the N/C interaction by high-affinity AR agonists appears to contribute to their biological potency at low physiological concentrations. Lack of induction of the N/C interaction may account for the high MPA concentrations required to stabilize AR, which would contribute to a reduced biological potency as an androgen agonist.

The apparent discrepancy in ligand potency between maximal induction in the N/C assay using the GAL4-AR carboxyl-terminal and the VP16-AR NH₂-fragment fusion proteins (0.1–1 nM) vs. agonist potency of full-length AR in the luciferase assay (0.001–0.01 nM) probably reflects deletion of the NH₂-terminal domain. We demonstrated previously that although the AR ligand-binding domain retains high-affinity binding after deletion of the NH₂-terminal region, the ligand dissociation rate increases 5- to 7-fold, and

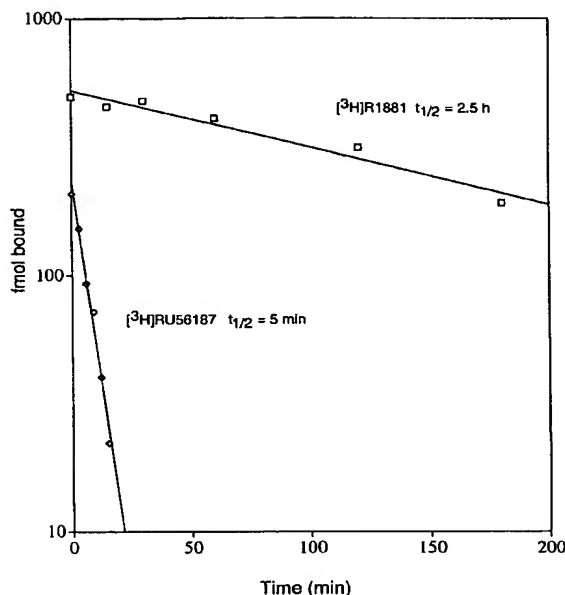


Fig. 9. Dissociation Rates of $[^3\text{H}]\text{R1881}$ and $[^3\text{H}]\text{RU56187}$ from Human AR

COS cells were transfected with pCMVhAR and incubated with 5 nM $[^3\text{H}]\text{R1881}$ or 5 nM $[^3\text{H}]\text{RU56187}$ for 2 h at 37°C followed by the addition of a 10,000-fold molar excess of unlabeled ligands as described in *Materials and Methods*. Cells were harvested at increasing time intervals, and radioactivity was determined. The data are shown on a semilog plot, and the half-time of dissociation is indicated: $t_{1/2}$ 2.5 \pm 0.5 h for $[^3\text{H}]\text{R1881}$ and $t_{1/2}$ 5 \pm 1 min for $[^3\text{H}]\text{RU56187}$.

androgen no longer stabilizes this truncated receptor as it does full-length AR (22). Thus, higher ligand concentrations are likely required for the N/C interaction between AR fragments than between monomers of full-length AR.

Equilibrium dissociation constants (MPA, 1.7–2.9 nM; DHT, 0.9–2.6 nM) and saturation binding capacities (MPA, 107–249 fmol/mg protein; DHT, 42–257 fmol/mg protein) for MPA and DHT binding to AR were similar when measured in rat pituitary and hypothalamic extracts (28). However, direct measurement of *in vivo* bioactivity classifies MPA as a weak androgen. MPA increases the synthesis of β -glucuronidase in mouse kidney but only at 100-fold higher doses relative to testosterone (11, 12). In the androgen-insensitive Tfm mouse, β -glucuronidase activity did not increase, indicating that gene activation in response to MPA is AR mediated (12). MPA doses up to 1000 times higher than testosterone were required to increase ventral prostate weight in castrated rats (13). High-dose (0.9 mg/day) MPA was less effective than low-dose DHT (0.2 mg/day) in stimulating the synthesis of rat prostatic binding protein mRNA, and the effects of MPA were inhibited by flutamide (19), again indicating that its *in vivo* activity is AR mediated. MPA, rather than a metabolite, was shown to bind AR, and its low

in vivo androgenic activity correlated with reduced nuclear uptake (29, 30). MPA was reported to dissociate rapidly from AR (13, 31), although these studies did not account for possible degradation of the MPA-AR complex. X-ray crystal analysis indicates that MPA has an inverted $1\beta,2\alpha$ half-chair conformation of the A-ring resulting from steric strain by the 6α -methyl group that restricts side chain flexibility (32). This predicted rigid structure of MPA is in contrast to the flat, flexible structure of methyltrienolone (R1881), which can undergo large shape changes (33). It is conceivable that ligand flexibility facilitates the conformational changes required for the AR N/C interaction.

Acetate derivatives of steroids often have slower metabolic breakdown rates, making them candidates for use in hormone therapy (34). MPA, available for clinical use as Provera or Depo-Provera, has been used in the treatment of sexual precocity; its progestin and weak androgen effects inhibit pituitary gonadotropin secretion and lower gonadal steroid production (35). Stimulation of the growth of pubic hair in female patients without a significant slowdown in skeletal maturation (35) suggested weak androgenic activity of large doses of MPA (200–300 mg every 7–10 days). 21-Hydroxylated metabolites of MPA bind the glucocorticoid receptor and suppress pituitary secretion of ACTH. Because of its progestational effect, MPA was formerly used to prevent spontaneous abortion. However, prenatal exposure to MPA was reported to cause mild clitoral hypertrophy and posterior labial fusion in the female and hypospadias in the male (36–39), contraindicating its use during pregnancy. The virilizing effect of MPA in the female fetus can be explained by its androgenic activity. Antiandrogen effects of MPA in the male fetus could result from competition for DHT binding to AR and subsequent insufficient agonist activity. Anogenital distance, a measure of antiandrogen activity in rodents (1), was lengthened in females and shortened in males exposed to MPA during fetal development. More recently, MPA was approved for use in the United States as an injectable contraceptive based on its effectiveness in suppressing gonadotropin secretion, inhibiting follicular maturation and preventing ovulation. Doses of 150 mg im every 6 weeks to 3 months lack androgen effects in the adult female.

MPA has also been used in breast cancer therapy (40, 41). In an MFM-223 mammary cancer cell line that has high levels of AR, but low levels of estrogen, progesterone, and glucocorticoid receptors, cell proliferation was inhibited by 1 nM DHT or 10 nM MPA (42), indicating the AR agonist effect of MPA inhibits breast cancer cell growth. Response rates of breast cancer patients to MPA therapy correlated with higher AR levels (43). The antiproliferative activity of DHT and MPA on breast cancer cells was attributed to increased 17β -hydroxysteroid dehydrogenase activity, which promotes increased oxidation of estradiol to the weak estrogen, estrone (44).

Agonists vs. antagonist activity is influenced by metabolism, binding affinity, association and dissociation rates, and ligand-induced receptor conformation, stabilization, dimerization, DNA binding, and interactions with associating proteins. Clearly, equilibrium binding affinity is of limited usefulness in predicting *in vivo* bioactivity unless combined with measurements of ligand dissociation rates. AR binding affinity of RU56187 is similar to that for DHT, yet RU56187 is an antagonist *in vivo* (14, 15). The anabolic steroids, oxandrolone and fluoxymesterone, have high inhibition constants for binding, yet induce the N/C interaction and stabilize AR at relatively low ligand concentrations and are AR agonists *in vivo*. Oxandrolone induces male-specific liver P450 enzymes (45). Oxandrolone and fluoxymesterone are structurally related 17 α -alkylated synthetic anabolic steroids used clinically to promote weight gain, stimulate growth of the bone matrix, and improve libido and sexual performance (46). In low doses oxandrolone (47, 48) or fluoxymesterone (49) accelerate linear growth in children with constitutional growth delay and Turner's syndrome (50, 51).

Evidence from crystal structure analysis of the retinoic acid receptor- γ (52), thyroid hormone receptor (53), and estrogen receptor (54) indicates that hormone binding causes helix 12 [helix 11 in retinoid X receptor- α (55)] at the carboxyl terminus to undergo a conformational change closing down over the ligand-binding pocket. For the estrogen receptor, binding of the antagonist raloxifene prevents alignment of helix 12 over the binding pocket (54). MPA binding to AR may distort the position of helix 12 causing an increased rate of ligand dissociation and interference with the N/C interaction. Proper closure of helix 12 might be expected to slow ligand dissociation from the pocket and form a new interface for the N/C interaction. Alignment of helix 12 by MPA binding may differ from that induced by potent agonists or antagonists, a distortion that may account for the high MPA concentrations required to stabilize AR.

Part of the discrepancy between ligand binding affinity and agonist and antagonist activities relates to differences in ligand binding kinetics. Association and dissociation rate kinetics can be fast or slow for high-affinity ligands. Slow dissociation of the most potent AR agonists, DHT, mibolerone, and R1881, is associated with AR stabilization at low ligand concentration. Fast dissociating ligands such as RU56187 fail to stabilize AR and have agonist activity in transcriptional activation assays but are antagonists *in vivo*. Mutations in the AR hormone-binding domain at valine 889 and arginine 752 cause severe androgen insensitivity, increase the rate of dissociation of bound androgen without altering high-affinity equilibrium binding (22), disrupt the N/C interaction (6), and cause loss of AR stabilization by low ligand concentrations (22). These mutations likely increase the rate of ligand dissociation and AR degradation by preventing helix 12 from closing the binding pocket and interfering with the N/C interaction. Rapid ligand dissociation could reduce *in*

vivo agonist activity and enhance dose-dependent antagonist activity as suggested previously for some antiestrogens and antiandrogens (13).

The most reliable *in vitro* indicators of *in vivo* AR antagonist activity therefore appear to be failure of a ligand to stabilize AR against degradation at steroid concentrations of 500 nM or more and an inability to induce AR DNA binding. DNA binding itself, however, appears to be a poor indicator of agonist potency. *In vivo* agonist activity is best reflected by a slow dissociation rate of bound ligand, AR stabilization at low ligand concentrations (≤ 10 nM), and induction of the N/C interaction. Concentrations at which a ligand activates AR in MMTV-luciferase assays can indicate agonist potency. MPA is an agonist in transient transcription assays but requires 100-fold higher concentrations than DHT. A similar shift in *in vitro* sensitivity to DHT results from certain AR missense mutations that cause partial or complete androgen insensitivity (56), indicating the critical importance of AR activation by low ligand concentrations. However, even though acetate derivatives of steroids have increased metabolic half-lives, it cannot be ruled out that the *in vivo* pharmacology of MPA limits its bioavailability to the AR.

A model for androgen-induced AR dimerization suggests an antiparallel orientation of monomers interacting through the DNA-binding domain and a ligand-dependent N/C interaction (4). Similar studies with the estrogen receptor (5) and AR fragments expressed in yeast (57) predict a parallel interaction model, and studies on solution dimerization of the human progesterone receptor favor a parallel model (58). More recent studies on AR made use of androgen insensitivity mutations in the steroid-binding domain that do not interfere with high-affinity equilibrium binding of androgen but increased the dissociation rate of bound androgen and disrupted the N/C interaction. Placement of the mutations in different AR fragments allowed assessment of directional dimerization in association with AR transcriptional activation, and the results were consistent with an antiparallel activated AR dimer model (6). Lack of an interaction between the ligand-binding domains bound to MPA or DHT argue against a parallel dimer model for AR with either ligand. Taken together the results suggest that the N/C interaction is required for potent *in vivo* agonists to be effective at low concentrations, but is not required for AR DNA binding *in vitro* or weak *in vivo* agonist activity at higher ligand concentrations. Formation of the N/C interaction likely contributes to *in vivo* potency by stabilizing AR at low ligand concentrations.

MATERIALS AND METHODS

Ligand Binding and Dissociation

Reagents were obtained as previously reported (22) with MPA and other steroids from Sigma Chemical Co. (St. Louis, MO) and RU56187 from Roussel Uclaf. Relative equilibrium

binding was determined in COS cell competitive binding assays using [³H]R1881. Monkey kidney COS cells (3.5×10^5 cells per well of six-well plate) were transiently transfected using diethylaminoethyl (DEAE)-dextran and 1 μ g pCMVhAR full-length AR expression vector per well. Cells were maintained in 10% calf serum and DMEM for 36 h and labeled for 2 h at 37 C with 5 nM [³H]R1881 in the presence and absence of increasing concentrations of unlabeled ligands. Cells were washed with PBS and harvested in 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8, and radioactivity was determined by scintillation counting. Dissociation rate kinetics were determined in COS cells transfected as described above using 3 μ g pCMVhAR/well. Transfected cells were incubated with 5 nM [³H]R1881 for 2 h followed by the addition of a 10,000-fold molar excess of unlabeled ligand. After increasing times, cells were washed with PBS and harvested in 0.5 ml of the SDS buffer above. Radioactivity was determined by scintillation counting. Apparent inhibition constants (K_i) for hydroxyflutamide, oxandrolone, and fluoxymesterone were determined using rat prostate cytosols prepared from tissue obtained 24 h after castration, extracted in binding buffer as previously described (1, 59), and incubated for 20 h at 4 C with 0.5–20 nM [³H]R1881 with or without increasing concentrations of unlabeled ligands between 0.1 and 1 μ M. Apparent K_i values were determined using double reciprocal plots and slope-replot analysis.

N/C Luciferase Assay

Recombinant fusion proteins included GALD-H which contained the *Saccharomyces cerevisiae* GAL4 DNA-binding domain amino acid residues 1–147 linked in frame with human AR steroid-binding domain amino acid residues 624–919. VPAR1–660 contained the herpes simplex virus VP16 trans-activation domain amino acid residues 411–456 linked in frame with AR NH₂-terminal and DNA-binding domain amino acid residues 1–660 (4). CHO cells (0.4×10^6 cells per 6-cm dish) were transfected using DEAE-dextran and 1 μ g GALD-H, 1 μ g VPAR1–660, and 5 μ g G5E1b-luciferase per plate, the latter containing five GAL-4 DNA-binding sites (60). DNA was added to 0.42 ml H₂O plus 0.5 ml 2 \times TBS (0.14 M NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.9 mM NaH₂PO₄, and 25 mM Tris-HCl, pH 7.4), and then 0.11 ml DEAE-dextran (0.5%) was added, after which the mixture was added to the aspirated plates and incubated for 1 h at 37 C. Plates were aspirated and 4 ml α -MEM containing 10% calf serum, penicillin/streptomycin, and 20 mM HEPES, pH 7.2, were added and incubated at 37 C for 3 h followed by a 4-min 15% glycerol shock in α -MEM. Cells were washed twice with 4 ml TBS, and 4 ml 0.2% calf serum- α -MEM media were added. The medium was changed 24 and 48 h later to serum-free medium and ligands were added. Cells were washed 4 h after the last addition with 4 ml PBS and harvested in 0.5 ml lysis buffer (Ligand Pharmaceuticals Inc., San Diego, CA). Luciferase light units were measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). To test for inhibitory activity, cells were incubated with 1 nM DHT in the presence of increasing concentrations of ligands. Decreases due to toxicity of up to 1 μ M ligand were monitored using an AR fusion plasmid GAL-A1 (4) coding for a constitutively active fusion protein containing AR NH₂-terminal residues 1–503 linked to the GAL-4 DNA-binding domain. Decreases in constitutive transcriptional activity after exposure were minimal.

MMTV-Luciferase Assay

Monkey kidney CV1 cells (0.4×10^6 cells per 6-cm dish) were transfected 24 h after plating using calcium phosphate with 100 ng human AR expression vector pCMVhAR and 5 μ g MMTV-luciferase reporter vector per plate. DNA is added to 0.28 M NaCl, 1.5 mM Na₂HPO₄, 0.05 M HEPES, pH 7.11 (0.14

ml/plate; 0.28 M NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.12; 0.25 M CaCl₂ (0.14 ml/plate) was added dropwise with vortexing and incubated 5 min, followed by the addition of DMEM-H media containing 10% calf serum, penicillin, streptomycin, and 20 mM HEPES, pH 7.2 (0.8 ml/plate) to minimize particle size, and then incubated for 15 min. The mixture was added to the aspirated plates followed by the addition of 3 ml DMEM-H containing 10% calf serum and incubated for 4 h at 37 C. Cells are washed twice with TBS, and 4 ml phenol red-free medium containing 0.2% calf serum was added. Ligands were added and cells were harvested and assayed as described above for the N/C luciferase assay.

DNA Mobility Shift Assay

Spodoptera frugiperda (Sf9) cells plated at 3.5×10^6 cells per 6-cm dish or 1×10^7 cells per 10-cm dish were infected for 45 h at 27 C at multiplicity of infection of 1–5 with AR recombinant baculovirus in *Autographa californica* nuclear polyhedrosis virus (AcMNPV) coding for full-length human AR, AR1–660 coding for the NH₂-terminal, DNA-binding, and hinge regions (amino acid residues 1–660), and AR507–919 coding for the DNA- and steroid-binding domains (amino acid residues 507–919) (25). The indicated concentrations of ligands were added 24 h and again 4 h before cell harvest. Cells were washed once in PBS at 4 C, pelleted, and resuspended in 0.15 ml/6-cm dish or 0.4 ml/10-cm dish in high-salt extraction buffer containing 0.5 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM Tris, pH 7.4 with protease inhibitors, 40 μ M leupeptin, 5 μ M aprotinin, 10 μ M pepstatin A, 2 mM Pefabloc, 5 mM benzamide, and 10 mM ϵ -amino-*n*-caproic acid. Cells were frozen and thawed three times, incubated on ice for 40 min, and microfuged for 15 min. Supernatants were dialyzed against the above buffer except containing 25 mM KCl and 0.5 mM phenylmethylsulfonyl fluoride as the only protease inhibitor. The reaction mixture contained approximately 20 μ g total cell protein of either full-length AR, AR1–660 (N), or AR507–919 (C), or when combined, 10 μ g total protein each for extracts of N and C. The reaction mix also contained 4 μ g of poly dI-dC, 80 μ g BSA, and DNA-binding buffer (25 mM KCl, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5) to a final volume of 20 μ l. ³²P-labeled oligonucleotides (0.2–0.3 ng, 20,000–25,000 cpm) were added and incubated for 1 h on ice. Annealing oligos 5'-CGACCAGAGTACGTGATGTTCTCAGG-3' with AccI-5' compatible end and 5'-GATCCCTGAGAACAT-CACGTACTCTGGT-3' with 3' BamHI compatible end were ³²P-labeled using the Klenow fragment of DNA polymerase. The androgen response element (underlined) derives from the 0.5-kb first intron fragment of the rat C3 prostatein gene (61). Before electrophoresis, 2 μ l 0.2% bromophenol blue were added, and the 5% nondenaturing acrylamide gel was pre-electrophoresed at 100 V for 30 min at 4 C. Samples are electrophoresed at 150 V for 4 h at 4 C. Gels are dried under vacuum at 80 C for 1 h and exposed to Biomax MR x-ray film (Eastman Kodak, Rochester, NY) at –80 C.

AR Stabilization

Full-length AR was expressed from pCMVhAR (8 μ g) in COS cells (1.2×10^6 cells/10-cm dish) transfected using DEAE-dextran. After 48 h, cells were incubated in methionine-free medium for 20 min followed by the addition of methionine-free medium containing 100 μ Ci [³⁵S]L-methionine/cysteine (PRO-MIX, Amersham, >1000 Ci/mmol) *in vitro* labeling mix. Cells were incubated for increasing times in the presence of the indicated concentrations of ligands, washed twice with PBS, and harvested in RIPA buffer and immunoprecipitated using AR52 antipeptide AR antibody as previously described (22).

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The Effects of Transdermal Dihydrotestosterone in the Aging Male: A Prospective, Randomized, Double Blind Study

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The objective of the study was to investigate the effects of dihydrotestosterone (DHT) gel on general well-being, sexual function, and the prostate in aging men. A total of 120 men participated in this randomized, placebo-controlled study (60 DHT and 60 placebo). All subjects had nocturnal penile tumescence once per week or less, andropause symptoms, and a serum T level of 15 nmol/liter or less and/or a serum SHBG level greater than 30 nmol/liter. The mean age was 58 yr (range, 50–70 yr). Of these subjects, 114 men completed the study. DHT was administered transdermally for 6 months, and the dose varied from 125–250 mg/d. General well-being symptoms and sexual function were evaluated using a questionnaire, and prostate symptoms were evaluated using the International Prostate Symptoms Score, transrectal ultrasonography, and assay of serum prostate-specific antigen.

Early morning erections improved transiently in the DHT group at 3 months of treatment ($P < 0.003$), and the ability to

maintain erection improved in the DHT group compared with the placebo group ($P < 0.04$). No significant changes were observed in general well-being between the placebo and the DHT group. Serum concentrations of LH, FSH, E2, T, and SHBG decreased significantly during DHT treatment. Treatment with DHT did not affect liver function or the lipid profile. Hemoglobin concentrations increased from 146.0 ± 8.2 to 154.8 ± 11.4 g/liter, and hematocrit from $43.5 \pm 2.5\%$ to $45.8 \pm 3.4\%$ ($P < 0.001$). Prostate weight and prostate-specific antigen levels did not change during the treatment. No major adverse events were observed.

Transdermal administration of DHT improves sexual function and may be a useful alternative for androgen replacement. As estrogens are thought to play a role in the pathogenesis of prostate hyperplasia, DHT may be beneficial, compared with aromatizing androgens, in the treatment of aging men. (*J Clin Endocrinol Metab* 87: 1467–1472, 2002)

ANDROGEN PRODUCTION declines with age in men, resulting in decreased serum concentrations of both total and bioavailable T (1–3). In healthy men, bioavailable free T declines by approximately 1%/yr between 40 and 70 yr (3) and by even more in unhealthy groups. Furthermore, the circadian rhythmicity of blood total T concentrations decreases with age (4). In contrast to menopausal symptoms in women, these age-related changes in testicular function are gradual, and the clinical picture may be difficult to recognize. However, a number of changes typically experienced by aging males have been attributed to a decline in circulating T levels. The symptoms are diminished energy, virility, and fertility and decrease in bone and muscle mass associated with an increase in adiposity (1–3, 5).

Improvement of clinical symptoms of andropause via androgen substitution therapy has long been recognized (6, 7). A number of androgen preparations have been tested to see whether androgen replacement could improve physical and mental well-being in aging men. T, the most frequently used androgen, has been administered orally, by injection, and recently via transdermal patches in hypogonadal men and in men suffering from andropause symptoms (8). Dehydroepiandrosterone has also been used for androgen replacement therapy,

and it has been shown to improve well-being in both aging women and men (9). More recently, percutaneous dihydrotestosterone (DHT) gel has become available as a method of androgen replacement (10). DHT, which cannot be aromatized to E2, may have advantages compared with aromatizing androgens (10). As E2 is thought to play a role in the pathogenesis of benign prostate hyperplasia, the treatment of andropause symptoms with nonaromatizing DHT may offer an advantage compared with aromatizing androgens. Furthermore, based on bioassay studies, DHT may have greater pharmacological potency than other available androgens (11).

To assess the efficacy and safety of DHT in the treatment of andropause symptoms we administered DHT gel or placebo transdermally to 120 men for 6 months, in a double blind, placebo-controlled monocenter study. In addition to andropause symptoms, special attention was paid to prostate tolerance, hematological parameters, and the lipid profile.

Subjects and Methods

Subjects

A total of 120 males, aged 50–70 yr (mean age, 58 yr), participated in this monocenter, double blind, randomized, placebo-controlled, parallel group study (Fig. 1, flow chart). Based on a telephone conversation or a clinic visit, 178 subjects were known to fulfill the symptom criteria, and they were asked to come for screening. Of them, 55 failed to enter the study because of abnormal lipid or liver parameters, high serum prostate-specific antigen (PSA; >10 $\mu\text{g/liter}$), or other reasons. The subjects

Abbreviations: DHT, Dihydrotestosterone; FAI, free androgen index; Hb, hemoglobin; Hcr, hematocrit; I-PPS, International Prostate Symptoms Score; PSA, prostate-specific antigen.

were randomized to the DHT (n = 60) or the placebo (n = 60) group. The randomization codes identifying the treatment were kept in sealed envelopes and were broken only after all clinical and biochemical anal-

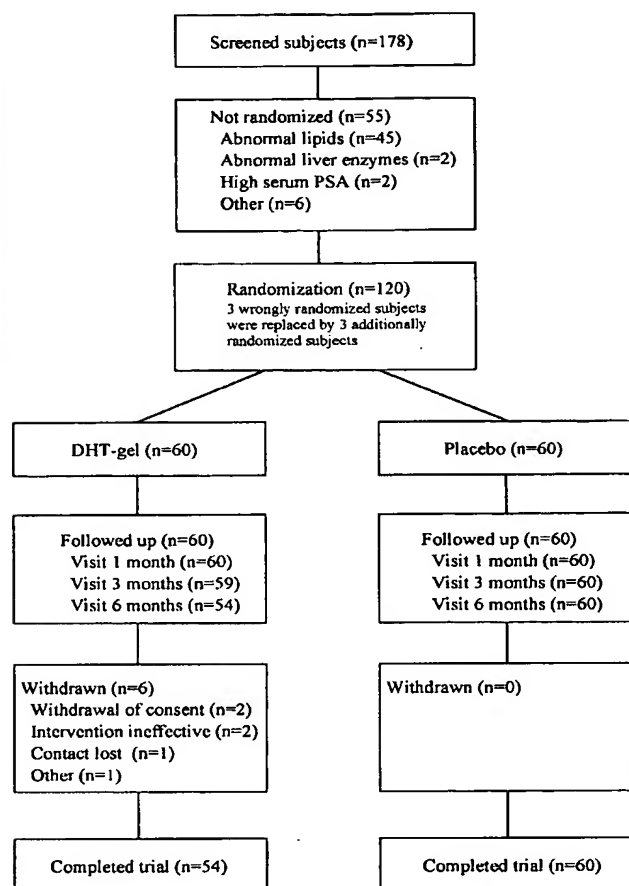


FIG. 1. Flow chart describing the progress of subjects throughout the study.

TABLE 1. Characteristics of the subjects

	All	DHT	Placebo	P value, DHT vs. PLACEBO
n	120	60	60	
Age (yr)	58.4 ± 5.3 ^a	58.3 ± 4.8	58.6 ± 5.7	NS
T ≤ 15 nmol/liter	51	25	26	NS
T < 9 nmol/liter	5	3	2	NS
SHBG > 30 nmol/liter	111	57	54	NS
SHBG > 62 nmol/liter	22	11	11	NS
T ≤ 15 nmol/liter and SHBG > 30 nmol/liter	43	22	21	NS
FAI	35.5 ± 10.8	35.3 ± 10.9	35.6 ± 10.7	NS
FAI (mean value in healthy men aged 20–49 yr)	76.9 ± 40.7 ^b			
FAI (mean value in healthy men aged 50–65 yr)	46.9 ± 16.4 ^b			
DHT (nmol/liter; mean value in healthy men aged 50–67 yr)	1.3 ± 0.7			

^a Mean ± SD.

^b P < 0.001 compared with the study subjects.

yses were completed. None of the envelopes had to be opened before completing the study. Subjects included should have had rarefaction of nocturnal penile tumescence (once or less per wk; frequency of early morning erections together with libido are known to have correlation with serum androgen levels) and at least one of the following andropause symptoms: decreased libido, erectile dysfunction, urinary disorders, asthenia, or depressive mood. In addition, the subjects had to have a total serum T concentration of 15 nmol/liter or less (normal range, 9–32 nmol/liter) and/or an SHBG level greater than 30 nmol/liter (normal range, 14–62 nmol/liter). Although serum T levels of 15 nmol/liter or less and SHBG levels greater than 30 nmol/liter do not define all subjects as being hypogonadal or having low free T, these limits were used together with clinical symptoms to find men who would benefit from the treatment. The number of subjects in each category is given in Table 1. Five subjects in the DHT group and nine in the placebo group (P = 0.175) had been treated earlier for impotence problems. The exclusion criteria with regard to prostate were prostate weight greater than 100 g, serum PSA level greater than 10 µg/liter, acute prostatitis, abnormal prostate in clinical or ultrasonographic examination, or prostatectomy/transurethral resection of the prostate. The other main exclusion criteria were significant cardiovascular disease, abnormal lipid profile (total cholesterol > 7.5 mmol/liter and/or triglycerides > 1.7 mmol/liter), alcohol abuse, and uncured cancer. Furthermore, subjects with neurological impotence, major depression, or other psychiatric diseases, and those taking hormones or drugs affecting sexual function, lipid/hormone metabolism (β-blockers, methylodopa, clonidine, guanethine thiazide diuretics, spironolactone, digitalis, barbiturates, clofibrate, cimetidine, metochlopramide, or antidepressive and neuroleptic drugs), or hematological parameters were excluded. Three subjects (1 taking DHT: unstable hypertension; 2 taking placebo: coronary heart disease with metoprolol medication and skin cancer) were wrongly included in the study; therefore, 3 additional men were randomized. Six men in the DHT group dropped out before the end of the trial (Fig. 1). The reasons for drop-out were withdrawal of consent (n = 2), lack of efficacy (n = 2), contact lost (n = 1), and acute pyelonephritis due to prostatitis (n = 1). For comparison, the serum concentrations of DHT in 35 healthy men, aged 50–67 yr, and the free androgen index (FAI) in 146 healthy men, aged 20–65 yr, were analyzed.

The study was approved by the ethics committee of the University of Oulu, and all subjects signed an informed consent form. The subjects could discontinue the study any time, and a serious adverse event was considered an absolute stopping rule.

Protocol

DHT and placebo gel were prepared and packed in identical tubes by Laboratories Besins Iscovesco (Paris, France). Both the study drug and placebo were opalescent gels with alcoholic odor, and they were applied on upper arms/shoulders and on abdomen if necessary. The DHT gel contained a 2.5% solution of DHT. After application the gel dried rapidly in a few minutes. The subjects were asked to wash their hands after

application and to pay attention to any skin irritation observed. All subjects administered 5 g DHT (125 mg DHT) or placebo gel daily for the first 30 d, whereafter the dose was adjusted by a outside person (*i.e.* blind to the principal investigators) on the basis of serum DHT measurement performed 20 d after study entry. If serum DHT was less than 5.8 nmol/liter, the men used a daily dose of 250 mg, if serum DHT was between 5.8–11.6 nmol/liter, the daily dose was 187.5 mg, and if serum DHT was over 11.6 nmol/liter, the daily dose was 125 mg. The purpose was to reach the upper limit of 5.8–11.6 nmol/liter, which has been found to be the range at which a daily dose of 5 g gel is used (12). The dose of placebo was adjusted randomly by an outside person; 30 subjects continued with 5 g, and 30 used either 7.5 or 10 g. All tubes were returned and weighed to ensure compliance, and no significant failures were observed. The effect of DHT on general well-being was evaluated by questionnaire (13 questions), which was modified from the Psychological General Well-Being scale (13), and 12 questions regarding sexual function were modified from the International Index of Erectile Function (14). For example, the scoring system for the early morning erections was: 1 = never, 2 = every other month, 3 = every month, 4 = every other wk, 5 = every week, 6 = two times a week or more. After the screening visit and entry, follow-up visits were made at 1, 3, and 6 months of treatment, and the subjects filled out the questionnaire before entry and at 3 and 6 months.

The prostate was palpated, and serum PSA was assayed at each visit. Transrectal ultrasonography of the prostate (Brüel & Kjaer Medical 3535, transducer 8551, 7 MHz, Naerum, Denmark) was carried out in three dimensions at the beginning of the study and at the last visit. Urinary symptoms were evaluated using the International Prostate Symptoms Score. Blood samples for T, E2, FSH, and SHBG measurements were drawn at 0 and 6 months and at each visit for other hormonal, hematological and biochemical analyses. All blood samples were taken after an overnight fast.

Laboratory techniques

Serum DHT concentrations were measured by RIA after organic extraction and hydrophobic chromatography. The lower limit of quantification of serum DHT was 0.1 nmol/liter. The normal range for DHT was 1–10 nmol/liter, and the intra- and interassay coefficients of variation were 9.1% and 6.6%, respectively. In previous studies the basal serum levels of DHT have been found to be 1.5–2.0 nmol/liter in men between 50–70 yr of age (5, 15, 16) and 2.5–3.5 nmol/liter in men between 19–29 yr of age (16). Serum T concentrations were measured using a ACS:180 chemiluminescence system with an ACS:180 analyzer (Chiron Corp., Emeryville, CA). Serum E2 concentrations were measured by RIA (Orion Diagnostica, Turku, Finland). Serum SHBG, LH, FSH and PSA concentrations were quantified by two-site fluorimmunoassay methods with kits obtained from Wallac, Inc. (Turku, Finland), using a 1235 AutoDELFLIA automatic immunoassay system. The intra- and interassay coefficients of variation were 4.0% and 5.6% for T, 5.7% and 6.4% for E2, 1.3% and 5.1% for SHBG, 4.9% and 6.5% for LH, 3.8% and 4.3% for FSH, and 1.2% and 3.8% for PSA, respectively. The FAI was calculated according to the equation: $(T \times 100)/SHBG$. Hematological analyses and biochemical measurements were performed using approved routine clinical chemistry methods (Oulu University Hospital).

Statistics

The homogeneity of the two groups before inclusion and before treatment was analyzed using a *t* test in normally distributed variables (placebo, *n* = 60; DHT, *n* = 60). Wilcoxon's nonparametric test was used for variables with persisting skewed distribution, and χ^2 or Fisher exact test was used for qualitative variables. For comparison of main efficacy and biochemical parameters the repeated measures ANOVA was used (placebo, *n* = 60; DHT, *n* = 54).

Results

After 1 month of DHT treatment, 23% of the subjects used a daily dose of 125 mg, 45% used 187.5 mg, and 32% used 250 mg. The serum concentrations of DHT and other hormones are shown in Table 2. For comparison, the FAI and serum

TABLE 2. Effects of DHT treatment on serum hormone parameters

Hormone	DHT			Placebo			P value, mean change DHT vs. placebo
	0 month	3 months	6 months	0 month	3 months	6 months	
DHT (nmol/liter (range))	1.5 ± 0.6 (0.5–3.3)	9.3 ± 4.7 (1.6–24.8)	8.2 ± 4.6 (1.5–24.5)	1.5 ± 0.8 (0.1–5.0)	1.4 ± 0.8 (0.2–4.4)	1.5 ± 0.7 (0.2–4.2)	<0.001
Dose of DHT, 125 mg (5 g gel)	1.5 ± 0.7 (n = 14)	9.5 ± 6.9	8.5 ± 6.5	1.6 ± 0.9 (n = 30)	1.5 ± 1.0	1.6 ± 0.9	<0.001
Dose of DHT, 187.5 mg (7.5 g gel)	1.5 ± 0.6 (n = 27)	9.0 ± 3.9	8.2 ± 4.8	1.4 ± 0.5 (n = 22)	1.4 ± 0.6	1.4 ± 0.5	<0.001
Dose of DHT, 250 mg (10 g gel)	1.5 ± 0.7 (n = 19)	9.2 ± 4.1	8.0 ± 2.8	1.2 ± 0.3 (n = 8)	1.0 ± 0.4	1.2 ± 0.4	<0.001
T (nmol/liter)	16.1 ± 4.6		5.9 ± 3.9	15.9 ± 4.5		15.3 ± 5.1	<0.001
E2 (nmol/liter)	0.09 ± 0.03		0.05 ± 0.02	0.08 ± 0.02		0.09 ± 0.04	<0.001
SHBG (nmol/liter)	48.9 ± 17.3		38.7 ± 12.9	46.5 ± 16.6		42.7 ± 16.7	0.003
FSH (IU/liter)	6.5 ± 4.9		3.9 ± 3.8	5.7 ± 4.6		6.0 ± 5.3	<0.001
LH (IU/liter)	5.1 ± 2.9	2.4 ± 2.2	2.3 ± 1.8	4.5 ± 2.7	4.6 ± 3.1	4.6 ± 2.7	<0.001

DHT concentrations of healthy men are shown in Table 1. The score of early morning erection improved significantly in the DHT group during the first 3 months of treatment (from 3.0 to 3.9; $P < 0.003$). The ability to maintain erections in subjects taking DHT improved significantly compared with that in subjects using placebo (Table 3). There were no statistically significant differences in general well-being, libido, mood, or vitality between the groups. However, the placebo effect was statistically significant in several questions: mood, briskness, self confidence, depression, activity, cheerfulness, and relaxation improved in both groups; libido, general interest in everyday life, and energy in the placebo group; and satisfaction with sexual life in the DHT group. Serum PSA concentrations did not change during the treatment. Similarly, prostate size and International Prostate Symptoms Score (I-PPS) remained unchanged (Table 4).

DHT treatment decreased serum concentrations of E2, T, and SHBG ($P < 0.001$ – 0.003 ; Table 2). Similarly, serum concentrations of LH and FSH decreased in the DHT group compared with the placebo group. DHT treatment did not affect serum lipid parameters (Table 4). No changes were observed in liver enzymes. Hemoglobin (Hb) and hematocrit (Hcr) values increased significantly in the DHT group compared with the placebo group (Table 4). In the DHT group, six men had Hb between 170 and 180 g/liter at least once during the treatment (normal range, 135–170), and one subject had Hb of 184 g/liter and Hcr of 55% (normal range,

40–54%) at 3 months, but the values decreased to 176 g/liter and 53% at 6 months. There were no major clinical adverse events during DHT treatment. Three subjects experienced mild headache during DHT treatment compared with two subjects in the placebo group. None of the subjects described skin irritation during the treatment, but one subject in the DHT group had hair growth on the left shoulder and upper arm. Two subjects in both groups suffered from mild depression during the study. Other reported adverse events were not considered to be related to the treatment.

Discussion

This first placebo-controlled study carried out with DHT demonstrated a number of changes in both clinical and biochemical parameters in response to percutaneous DHT administration in men with relatively low bioavailable serum T levels and andropause symptoms. Treatment with DHT improved the ability to maintain erections and transiently improved early morning erections. However, these changes were small, though significant; therefore, their clinical importance remains uncertain. Six subjects in the DHT group and none in the placebo group dropped out before the end of the trial. We do not have a good explanation why only subjects in the DHT group stopped the study, but the difference in the dropout frequency between the groups was not statistically significant, and none of the reasons for dropping out were related to side-effects of the drug.

Androgens have been shown to have favorable consequences in the central nervous system by having a stimulating and maintaining effect on sexual function in men (17, 18). A number of androgen preparations have been used to treat hypogonadism (19). Treatment with percutaneous DHT gel increased serum total DHT levels 5-fold and led to concentrations that were clearly above the normal young adult male range (16). Although serum T concentrations decreased simultaneously with DHT administration by 50–70%, it is apparent that the total androgen effect increased signifi-

TABLE 3. Ability to maintain erection during intercourse

Treatment	0 month	3 months	6 months	<i>P</i> value, mean change DHT vs. placebo
Placebo	2.53 ± 1.44	2.65 ± 1.56	2.81 ± 1.56	0.04
DHT	2.26 ± 1.41	2.70 ± 1.50	3.24 ± 1.35	

Difficulties in maintaining erection during intercourse were scored from 1–6: 1, always; 2, in 75% of intercourses; 3, in 50%; 4, in less than 25%; 5, in less than 10%; and 6, never.

TABLE 4. Effects of DHT treatment on weight, prostatic weight, I-PPS score, and hematological, biochemical, and lipid parameters

Parameter	DHT				Placebo				<i>P</i> value, mean change DHT vs. PLA
	0 month	1 month	3 months	6 months	0 month	1 month	3 months	6 months	
Wt (kg)	79.5 ± 9.0		79.9 ± 8.8	79.5 ± 9.0	80.8 ± 9.2		81.1 ± 9.7	81.1 ± 9.3	NS
BMI	25.7 ± 2.1		25.8 ± 1.9	25.7 ± 1.9	26.1 ± 2.5		26.2 ± 2.6	26.1 ± 2.7	NS
Prostatic wt (g)	25.3 ± 10.0			25.9 ± 8.0	23.2 ± 7.8			25.5 ± 8.3	NS
I-PPS score	10.2 ± 6.3		8.6 ± 6.3	8.0 ± 5.3	8.0 ± 5.1		7.0 ± 5.7	6.9 ± 5.4	NS
Hematocrit (%)	43.5 ± 2.5	43.9 ± 2.8	46.0 ± 3.1	45.8 ± 3.4	43.4 ± 2.3	43.1 ± 2.5	42.9 ± 2.9	43.1 ± 3.0	<0.001
Red blood cells (10 ¹² /liter)	4.7 ± 0.3			5.1 ± 0.4	4.7 ± 0.3			4.6 ± 0.4	<0.001
Hemoglobin (g/liter)	146.0 ± 8.2	149.3 ± 9.8	155.9 ± 9.7	154.8 ± 11.4	146.3 ± 8.2	146.5 ± 8.7	146.2 ± 9.4	145.8 ± 9.7	<0.001
PSA (μg/liter)	1.6 ± 1.6	1.6 ± 1.6	1.6 ± 1.4	1.6 ± 1.4	1.5 ± 1.2	1.5 ± 1.2	1.5 ± 1.2	1.5 ± 1.3	NS
Total cholesterol (mmol/liter)	5.6 ± 0.7			5.7 ± 0.7	5.7 ± 0.7			5.5 ± 0.8	NS
HDL cholesterol (mmol/liter)	1.1 ± 0.2			1.2 ± 0.2	1.1 ± 0.3			1.2 ± 0.3	NS
Triglycerides (mmol/liter)	1.2 ± 0.3			1.7 ± 0.9	1.2 ± 0.3			1.5 ± 0.8	NS

cantly, especially because serum SHBG levels decreased simultaneously. This is supported by the observation that percutaneous T and DHT have an equal androgen effect in patients with hypogonadism (20), and DHT may have even greater pharmacological potency than other androgens (11). Androgen replacement in older men has been reported to increase the sense of well-being (21). In our study we did not find significant effects of DHT gel on well-being or vitality. The reason for this is not clear, but it is possible that many subjects had erectile dysfunction before the study and had great expectations for treatment. As impotence is often multifactorial, and androgen supplementation of older men has generally not met with great success (21, 22), at least some subjects may have been disappointed with the treatment and did not pay attention to general well-being. Furthermore, as in previous studies (21), the placebo effect in this study was significant with regard to several aspects of general well-being. In addition, based on the inclusion criteria ($T \leq 15$ nmol/liter and/or $SHBG > 30$ nmol/liter) the subjects may have had only mild or moderate androgen deficiency. Although the FAI of the study subjects was significantly lower than that of healthy men of the same age and was half that seen in younger men, their serum DHT levels at baseline were comparable. This was expected, because serum DHT levels do not change markedly with advancing age (5), and therefore the measurement of DHT levels may not be useful when considering androgen decline or deficiency in aging men. Furthermore, if some of the effects of androgens, for instance on the central nervous system, are due to metabolism to estrogens, DHT as a nonaromatizable androgen may have a weaker effect. Alternatively, the instruments used to assess subjective symptoms may not be sensitive enough to detect small changes, which is always a problem in studies like this.

Growth and function of the prostate are controlled by sex steroids. This is supported by the observations that neither prostate hyperplasia nor cancer occur in castrated men or young men (23, 24). Furthermore, androgen deprivation by way of a variety of agents has been shown to reduce prostate size in benign prostatic hyperplasia and to lead to regression of prostate cancer (25, 26). However, regression of prostate size has been described only when almost complete suppression of circulating (27) or tissue (28, 29) DHT is achieved. It has also been recognized, although not proved, that the use of T in elderly men may carry a potential risk by enhancing the progression of preclinical to clinical cancer. It is known that androgens stimulate the growth of clinically diagnosed prostate cancer. In some studies the use of physiological T enanthate supplementation is reflected in stimulation of PSA (19, 26), although in several other studies this has not been observed (30). Holmäng and associates (31) found that mean prostate volume increased by 12% during 8 months of treatment with T undecanoate. On the other hand, in many other studies no change in prostate volume during androgen treatment has been found (30). In our study serum PSA concentrations did not increase during DHT treatment, and prostate size remained unchanged. Nevertheless, it is recommended that men using androgen replacement therapy should be carefully screened and followed up periodically.

The mechanisms by which androgens affect the prostate

are not well established, but estrogens are thought to play a role. Experimental studies have shown the inability of non-aromatizable androgens to induce the early stage of prostate hypertrophy (32, 33). On the other hand, aromatizable androgens can induce prostate hyperplasia in monkeys, and this effect can be reversed with an aromatase inhibitor (34). Suzuki and associates (35) have shown that treatment with T combined with E2 stimulates more prostate growth in rats than T treatment alone. The results of a 1.8-yr survey of 37 men, aged 55–70 yr, treated with daily percutaneous DHT suggested that high serum levels of DHT effectively improved andropause symptoms while slightly, but significantly, reducing prostate size (10). In a previous study (20) as well as in the present study the administration of DHT decreased serum E2 levels by 50%. Although the above-mentioned findings and theories serve as a good basis with regard to the importance of estrogens in prostate function, further studies are needed to clarify the clinical significance of the decline of E2 concentrations during DHT treatment.

As expected and observed previously (15), serum FSH and LH concentrations decreased during DHT treatment as a result of the negative feedback effect. The long-term effect of DHT on testicular function, *e.g.* on spermatogenesis, is not yet known, but no evidence of irreversible effects exists (20).

It is well known that androgens have an anabolic effect. Androgens increase red cell mass and Hb concentrations mainly through a direct effect on erythropoietin synthesis in the kidneys, and inhibition of androgen secretion decreases Hb concentrations (36). We found that Hb increased significantly as early as after 1 month of DHT treatment. Similar effects on Hcr have been found earlier in long-term use of T in older hypogonadal men (19) and in normal aging males (31). This anabolic effect must be considered during the follow-up of subjects using DHT or other androgens; Hb and Hcr should be assessed after 3–6 months of treatment, and the dose should be decreased if necessary. Weight and body mass index did not change during the study. The possible effects of DHT on bone density as well as on body composition were not assessed, which may reduce the informativity of the study. These matters were considered carefully before the study, and because no significant changes, especially in bone density, were expected in 6 months of treatment they were not included.

Whether androgen therapy affects cardiovascular risk factors is not clear, although epidemiological studies have demonstrated higher risks in men with lower T levels (37, 38). Moreover, the effects of androgens on lipid metabolism are contradictory. Substitution of T in hypogonadal or T-deficient men has been associated with increases, decreases, or no change in serum high density lipoprotein levels (30, 39–43). In our study there were no changes in serum concentrations of total and high density lipoprotein cholesterol; overall the changes in serum lipids between DHT and placebo groups were modest, and the impact (*e.g.* risk or benefit of cardiovascular function) remains to be studied in long-term trials.

This study suggests that transdermal DHT gel may be a useful alternative for hormone replacement therapy in older men. Whether more significant improvement in sexual functions had been observed using higher doses of DHT remains

to be studied. DHT treatment resulted in hormonal changes that raise interesting questions about the significance of estrogens in prostate function. If estrogens play a role in prostate growth, as has been suggested, the use of nonaromatizable androgens may be beneficial compared with that of aromatizable androgens. Although no significant side-effects were noted, controlled follow-up trials of androgen replacement therapy in general are needed to clarify the possible long-term benefits and risks.

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[54] **METHODS FOR PREVENTING AND TREATING OSTEOPOROSIS WITH LOW DOSE NON-MASCULINIZING ANDROGENIC COMPOUNDS**

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[21] Appl. No.: **477,172**

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[52] U.S. Cl. **514/169; 514/170; 514/177; 514/179**

[58] Field of Search **514/169, 170, 514/177, 179**

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(List continued on next page.)

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[57] **ABSTRACT**

A method of treatment or prevention of breast and endometrial cancer, osteoporosis and endometriosis in susceptible warm-blooded animals comprising administering a low dose of a progestin or other steroid derivative having androgenic activity and low masculinizing activity. Pharmaceutical compositions useful for such treatment and pharmaceutical kits containing such compositions are disclosed. An in vitro assay permitting specific measurements of androgenic activity of potentially useful compounds is also disclosed.

26 Claims, 2 Drawing Sheets

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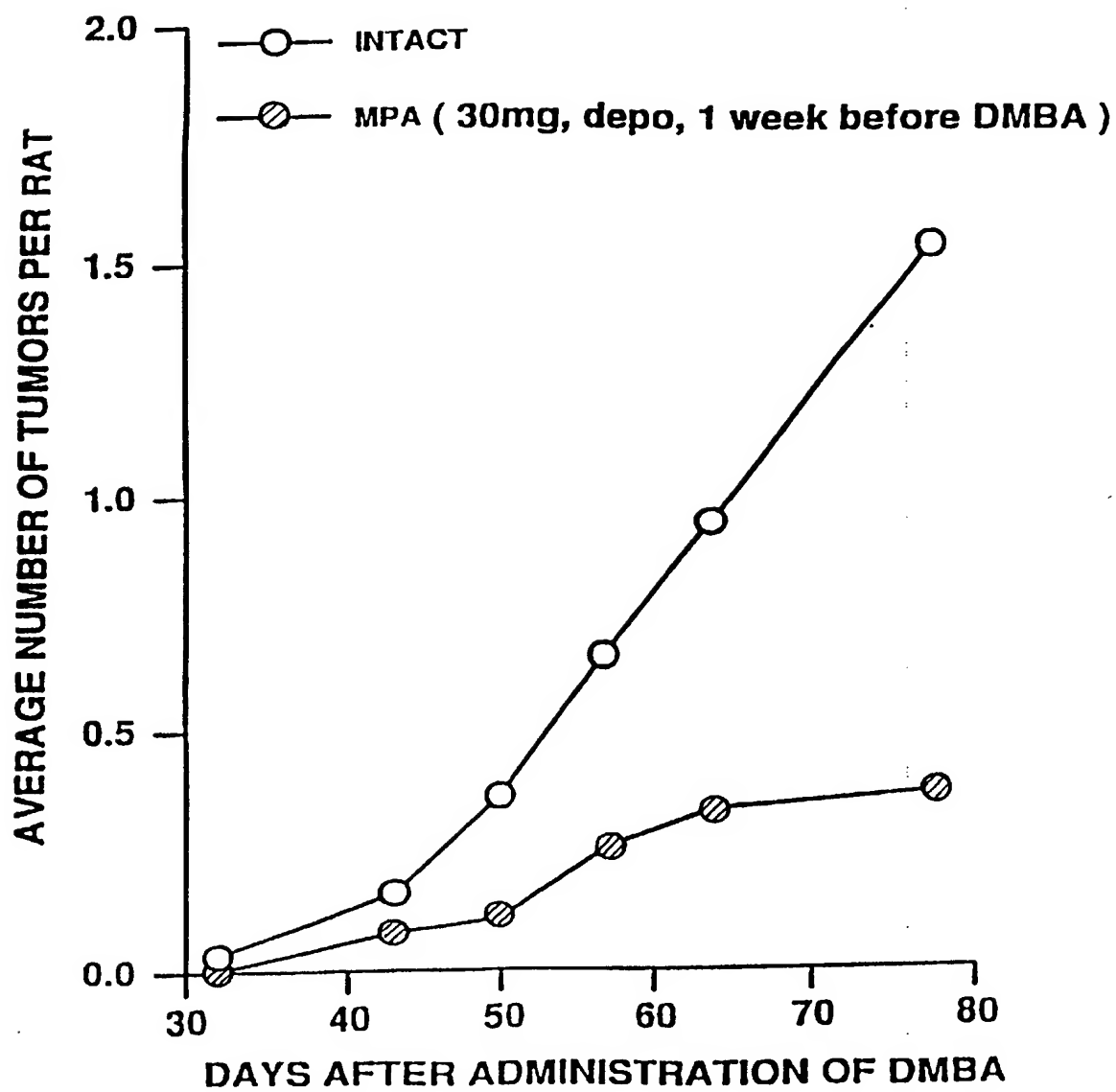


FIG. 1

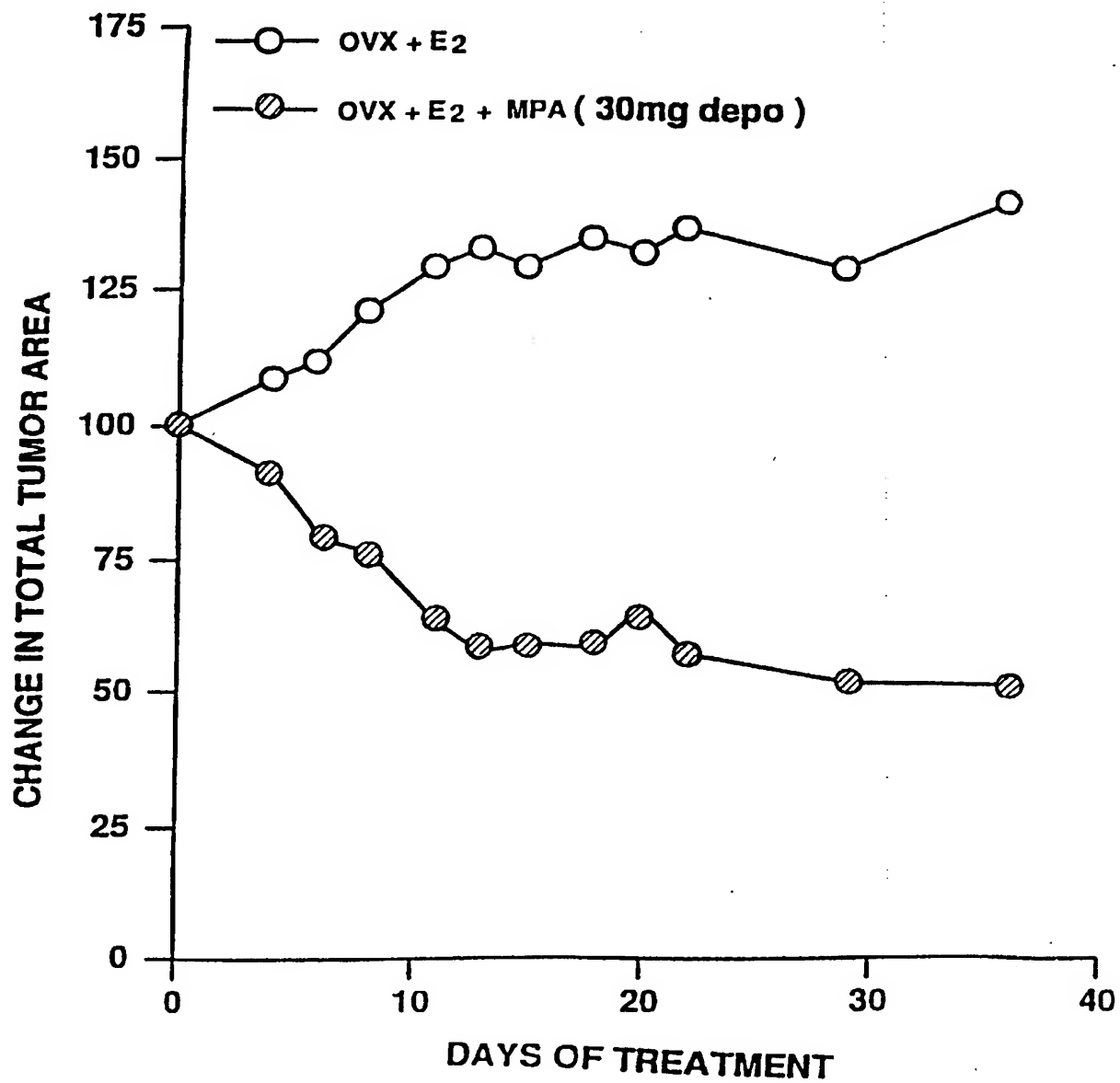


FIG. 2

METHODS FOR PREVENTING AND TREATING OSTEOPOROSIS WITH LOW DOSE NON-MASCULINIZING ANDROGENIC COMPOUNDS

This application is a division of U.S. patent application Ser. No. 08/282,964, filed Jul. 29, 1994, and now U.S. Pat. No. 5,545,634 which is in turn a division of U.S. patent application Ser. No. 08/015,083 filed Feb. 8, 1993, now U.S. Pat. No. 5,362,720 which is in turn a continuation of U.S. patent application Ser. No. 07/724,532 filed Jun. 28, 1991, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to a method for treating or preventing breast and endometrial cancer, bone loss, and for treating endometriosis in susceptible warm-blooded animals including humans involving administration of a compound possessing androgenic activity, and to kits containing active ingredients to be used in the therapy.

Various investigators have been studying hormonal therapy for breast and endometrial cancer as well as for the prevention and treatment of bone loss and for treatment of endometriosis. The main approaches for the treatment of already developed breast cancer are related to the inhibition of estrogen action and/or formation. The role of estrogens in promoting the growth of estrogen-sensitive breast cancer is well recognized (Lippman, *Semin. Oncol.* 10 (suppl. 4): 11-19, 1983; Sledge and McGuire, *Cancer Res.* 38: 61-75, 1984; Wittliff, *Cancer* 53: 630-643, 1984; Poulin and Labrie, *Cancer Res.* 46: 4933-4937, 1986).

Estrogens are also known to promote the proliferation of normal endometrium. Chronic exposure to estrogens unopposed by progesterone can lead to the development of endometrial hyperplasia which predisposes to endometrial carcinoma (Lucas, *Obstet. Gynecol. Surv.* 29: 507-528, 1974). The incidence of endometrial cancer increases after menopause, especially in women receiving estrogen therapy without simultaneous treatment with progestins (Smith et al., *N. Engl. J. Med.* 293: 1164-1167, 1975; Mack et al., *N. Engl. J. Med.* 294: 1262-1267, 1976).

Various investigators have been studying hormone-dependent breast and endometrial cancer. A known form of endocrine therapy in premenopausal women is castration most commonly performed by surgery or irradiation, two procedures giving irreversible castration. Recently, a reversible form of castration has been achieved by utilizing Luteinizing Hormone-Releasing Hormone Agonists (LHRH agonists) which, following inhibition of secretion of bioactive Luteinizing Hormone (LH) by the pituitary gland, decrease serum estrogens to castrated levels (Nicholson et al., *Brit. J. Cancer* 39: 268-273, 1979).

Several studies show that treatment of premenopausal breast cancer patients with LHRH agonists induces responses comparable to those achieved with other forms of castration (Klijn et al., *J. Steroid Biochem.* 20: 1381, 1984; Manni et al., *Endocr. Rev.* 7: 89-94, 1986). Beneficial effects of treatment with LHRH agonists have also been observed in postmenopausal women (Nicholson et al., *J. Steroid Biochem.* 23: 843-848, 1985).

U.S. Pat. No. 4,071,622 relates to the use of certain LHRH agonists against DMBA-induced mammary carcinoma in rats.

U.S. Pat. No. 4,775,660 relates to the treatment of female breast cancer by use of a combination therapy comprising administering an antiandrogen and an antiestrogen to a

female after the hormone output of her ovaries has been blocked by chemical or surgical means.

U.S. Pat. No. 4,775,661 relates to the treatment of female breast cancer by use of a therapy comprising administering to a female, after the hormone output of her ovaries has been blocked by chemical or surgical means, an antiandrogen and optionally certain inhibitors of sex steroid biosynthesis.

U.S. Pat. No. 4,760,053 describes a treatment of selected sex steroid dependent cancers which includes various specified combinations of compounds selected from LHRH agonists, antiandrogens, antiestrogens and certain inhibitors of sex steroid biosynthesis.

In U.S. Pat. No. 4,472,382 relates to treatment of prostatic adenocarcinoma, benign prostatic hypertrophy and hormone-dependent mammary tumors with specified pharmaceuticals or combinations. Various LHRH agonists and antiandrogens are discussed.

International Patent Application PCT/W086/01105, discloses a method of treating sex steroid dependent cancers in warm-blooded animals which comprises administering specific pharmaceuticals and combinations. Antiandrogens, antiestrogens, certain inhibitors of sex steroid biosynthesis and blocking of hormonal output are discussed.

The inventor's co-pending U.S. patent application Ser. No. 07/321926 filed Mar. 10, 1989, relates to a method of treatment of breast and endometrial cancer in susceptible warm-blooded animals which may include inhibition of ovarian hormonal secretion by surgical means (ovariectomy) or chemical means (use of an LHRH agonist, e.g. [D-Trp⁶, des-Gly-NH₂¹⁰]LHRH ethylamide, or antagonists) as part of a combination therapy. Antiestrogens, androgens, progestins, inhibitors of sex steroid formation (especially of 17 β -hydroxysteroid dehydrogenase- or aromatase-catalyzed production of sex steroids), inhibitors of prolactin secretion and of growth hormone secretion and ACTH secretion are discussed.

Androgen receptors have been shown to be present in normal (Wittliff, In: Bush, H. (Ed.), *Methods in Cancer Res.*, Vol. 11, Acad. Press, New York, 1975, pp. 298-304; Allegra et al., *Cancer Res.* 39: 1447-1454, 1979) and neoplastic (Allegra et al., *Cancer Res.* 39: 1147-1454, 1979; Engelsman et al., *Brit. J. Cancer* 30: 177-181, 1975; Moss et al., *J. Ster. Biochem.* 6: 743-749, 1975; Miller et al., *Eur. J. Cancer Clin. Oncol.* 2: 539-542, 1985; Lippman et al., *Cancer* 38: 868-874, 1976; Allegra et al., *Cancer Res.* 39: 1447-1454, 1979; Miller et al., *Eur. J. Clin. Oncol.* 21: 539-542, 1985; Lea et al., *Cancer Res.* 49: 7162-7167, 1989) as well as in several established breast cancer cell lines (Lippman et al., *Cancer Res.* 36: 4610-4618, 1976; Horwitz et al., *Cancer Res.* 38: 2434-2439, 1978; Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988). Androgen receptors are also present in dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in the rat (Asselin et al., *Cancer Res.* 40: 1612-1622, 1980).

Androgen receptors have also been described in human endometrium (MacLaughlin and Richardson, *J. Steroid Biochem.* 10: 371-377, 1979; Muechler and Kohler, *Gynecol. Invest* 8: 104, 1988). The growth inhibitory effects of the androgen methyltrienolone (R1881), on endometrial carcinoma in vitro have been described (Centola, *Cancer Res.* 45: 6264-6267, 1985).

Recent reports have indicated that androgen receptors may add to the selective power of estrogen receptors or even supplant estrogen receptors as best predicting response to endocrine therapy (Teulings et al., *Cancer Res.* 40: 2557-2561, 1980; Bryan et al., *Cancer* 54: 2436-2440, 1984).

The first androgen successfully used in the treatment of advanced breast cancer is testosterone propionate (Nathanson, *Rec. Prog. Horm. Res.* 1: 261-291, 1947). Many studies subsequently confirmed the beneficial effect of androgens on breast cancer (Alan and Herrman, *Ann. Surg.* 123: 1023-1035; Adair, *Surg. Gynecol. Obstet.* 84: 719-722, 1947; Adair et al., *JAMA* 140: 1193-2000, 1949). These initial results stimulated cooperative studies on the effect of testosterone propionate and DES which were both found to be effective in producing objective remissions. (Subcommittee on Steroid and Cancer of the Committee on Research of the Council on Pharmacy and Chemistry of the Am. Med. Association followed by the Cooperative Breast Cancer Group under the Cancer Chemotherapy National Service Center of the NCI who found that testosterone propionate improved remission rate and duration, quality of life and survival (Cooperative Breast Cancer Group, *JAMA* 188, 1069-1072, 1964)).

A response rate of 48% (13 of 27 patients) was observed in postmenopausal women who received the long-acting androgen methenolone enanthate (Kennedy et al., *Cancer* 21: 197-201, 1967). The median duration of survival was four times longer in the responders as compared to the non-responder group (27 versus 7.5 months). A large number of studies have demonstrated that androgens induce remission in 20 to 40% of women with metastatic breast cancer (Kennedy, *Hormone Therapy in Cancer. Geriatrics* 25: 106-112, 1970; Goldenberg et al., *JAMA* 223: 1267-1268, 1973).

A response rate of 39% with an average duration of 11 months has recently been observed in a group of 33 postmenopausal women who previously failed or did not respond to Tamoxifen (Manni et al., *Cancer* 48: 2507-2509, 1981) upon treatment with Fluoxymesterone (Halostatin) (10 mg, b.i.d.). Of these women, 17 had also undergone hypophysectomy. There was no difference in the response rate to Fluoxymesterone in patients who had previously responded to Tamoxifen and in those who had failed. Of the 17 patients who had failed to both Tamoxifen and hypophysectomy, 7 responded to Fluoxymesterone for an average duration of 10 months. Among these, two had not responded to either Tamoxifen or hypophysectomy.

The combination Fluoxymesterone and Tamoxifen has been shown to be superior to Tamoxifen alone. In fact, complete responses (CR) were seen only in the combination arm while 32% showed partial response (PR) in the combination arm versus only 15% in the monotherapy arm. In addition, there were only 25% of non-responders in the combination therapy arm versus 50% in the patients who received TAM alone (Tormey et al., *Ann. Int. Med.* 98:139-144, 1983). Moreover, the median time from onset of therapy to treatment failure was longer with Fluoxymesterone+Tamoxifen (180 days) compared to the Tamoxifen arm alone (64 days). There was a tendency for improved survival in the combination therapy arm (380 versus 330 days).

The independent beneficial effect of an androgen combined with an antiestrogen is suggested by the report that patients who did not respond to Tamoxifen could respond to Fluoxymesterone and vice versa. Moreover, patients treated with Tamoxifen and crossing over to Fluoxymesterone survived longer than those treated with the reverse regimen (Tormey et al., *Ann. Int. Med.* 98: 139-144, 1983).

Since testosterone propionate had beneficial effects in both pre- and post-menopausal women (Adair et al., *J. Am. Med. Ass.* 15: 1193-1200, 1949), it indicates that in addition

to inhibiting gonadotropin secretion, the androgen exerts a direct inhibitory effect on cancer growth.

Recent in vitro studies describe the relative antiproliferative activities of an androgen on the growth of the estrogen-sensitive human mammary carcinoma cell line ZR-75-1 (Poulin et al. "Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line", *Breast Cancer Res. Treatm.* 12: 213-225, 1989). As mentioned above, Poulin et al. (*Breast Cancer Res. Treatm.* 12: 213-225, 1989) have found that the growth of ZR-75-1 human breast carcinoma cells is inhibited by androgens, the inhibitory effect of androgens being additive to that of an antiestrogen. The inhibitory effect of androgens on the growth of human breast carcinoma cells ZR-75-1 has also been observed in vivo in nude mice (Dauvois and Labrie, *Cancer Res.* 51: 3131-3151, 1991).

As a possible mechanism of androgen action in breast cancer, it has recently been shown that androgens strongly suppress estrogen (ER) and progesterone (PgR) receptor contents in ZR-75-1 human breast cancer cells as measured by radioligand binding and anti-ER monoclonal antibodies. Similar inhibitory effects were observed on the levels of ER mRNA measured by ribonuclease protection assay. The androgenic effect is measured at subnanomolar concentrations of the non-aromatizable androgen 5 α -dihydrotestosterone, regardless of the presence of estrogens, and is competitively reversed by the antiandrogen hydroxyflutamide (Poulin et al., *Endocrinology* 125: 392-399, 1989). Such data on estrogen receptor expression provide an explanation for at least part of the antiestrogenic effects of androgens on breast cancer cell growth and moreover suggest that the specific inhibitory effects of androgen therapy could be additive to the standard treatment limited to blockade of estrogens by antiestrogens.

Dauvois et al. (*Breast Cancer Res. Treatm.* 14: 299-306, 1989) have shown that constant release of the androgen 5 α -dihydrotestosterone (DHT) in ovariectomized rats bearing DMBA-induced mammary carcinoma caused a marked inhibition of tumor growth induced by 17 β -estradiol (E₂). That DHT acts through interaction with the androgen receptor is supported by the finding that simultaneous treatment with the antiandrogen Flutamide completely prevented DHT action. Particularly illustrative of the potent inhibitory effect of the androgen DHT on tumor growth are the decrease by DHT of the number of progressing tumors from 69.2% to 29.2% in E₂-treated animals and the increase by the androgen of the number of complete responses (disappearance of palpable tumors) from 11.5% to 33.3% in the same groups of animals. The number of new tumors appearing during the 28-day observation period in E₂-treated animals decreased from 1.5 \pm 0.3 to 0.7 \pm 0.2 per rat during treatment with the androgen DHT, an effect which was also reversed by the antiandrogen Flutamide. Such data demonstrate, for the first time, that androgens are potent inhibitors of DMBA-induced mammary carcinoma growth by an action independent from inhibition of gonadotropin secretion and suggest an action exerted directly at the tumor level, thus further supporting the in vitro data obtained with human ZR-75-1 breast cancer cells (Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988).

The natural androgens testosterone (TESTO) and dihydrotestosterone (DHT) are formed from conversion of androstenedione into TESTO by 17 β -hydroxysteroid dehydrogenase and then TESTO into DHT by the action of the enzyme 5 α -reductase. The adrenal precursor 5-androst-5-ene-3 β ,17 β -diol can also be converted into TESTO by action of the enzyme 3 β -hydroxysteroid dehydrogenase/ $\Delta^5\Delta^4$ isomerase (3 β -HSD).

Since the natural androgens TESTO and DHT have strong masculinizing effects, numerous derivatives of TESTO as well as progesterone have been synthesized in order to obtain useful compounds having fewer undesirable masculinizing side effects (body hair growth, loss of scalp hair, acne, seborrhea and loud voice).

Medroxyprogesterone acetate (MPA) is one of the most widely used compounds in the endocrine therapy of advanced breast cancer in women (Mattsson, *Breast Cancer Res. Treatm.* 3: 231-235, 1983; Blumenschein, *Semin Oncol.* 10: 7-10, 1983; Hortobagyi et al., *Breast Cancer Res. Treatm.* 5: 321-326, 1985; Haller and Glick, *Semin. Oncol.* 13: 2-8, 1986; Horwitz, *J. Steroid Biochem.* 27: 447-457, 1987). The overall clinical response rate of high doses of this synthetic progestin averages 40% in unselected breast cancer patients (Horwitz, *J. Steroid Biochem.* 27: 447-457, 1987), an efficacy comparable to that of the non-steroidal antiestrogen tamoxifen (Lippman, *Semin. Oncol.* 10 (Suppl.): 11-19, 1983). Its more general use, however, is for breast cancer relapsing after other endocrine therapeutic modalities. The maximal inhibitory action of medroxyprogesterone acetate (MPA) on human breast cancer cell growth in vitro may be achieved at concentration as low as 1 nM while an approximately 1000-fold higher dose is often required for glucocorticoid action (Poulin et al., *Breast Cancer Res. Treatm.* 13: 161-172, 1989).

Until recently, the mechanisms underlying the antitumor activity of MPA were poorly understood and have been attributed to interaction with the progesterone receptor. This steroid, however, presents a high affinity for progesterone (PgR) as well as for androgen (AR) and glucocorticoid receptors (GR) in various animal tissues (Perez-Palacios et al., *J. Steroid Biochem.* 19: 1729-1735, 1983; Janne and Bardin, *Pharmacol. Rev.* 36: 35S-42S, 1984; Pridjian et al., *J. Steroid Biochem.* 26: 313-319, 1987; Ojasso et al., *J. Steroid Biochem.* 27: 255-269, 1987) as well as in human mammary tumors (Young et al., *Am. J. Obstet. Gynecol.* 137: 284-292, 1980), a property shared with other synthetic progesterone derivatives (Bullock et al., *Endocrinology* 103: 1768-1782, 1978; Janne and Bardin, *Pharmacol. Rev.* 36: 35S-42S, 1984; Ojasso et al., *J. Steroid Biochem.* 27: 255-269, 1987). It is known that in addition to progesterone receptors (PgR), most synthetic progestational agents bind with significant affinity to androgen (AR) as well as glucocorticoid (GR) receptors, and induce biological actions specifically determined by these individual receptor systems (Labrie et al., *Fertil. Steril.* 28: 1104-1112, 1977; Engel et al., *Cancer Res.* 38: 3352-3364, 1978; Raynaud et al., In: *Mechanisms of Steroid Action* (G. P. Lewis, M. Grisburg, eds), MacMiland Press, London, pp. 145-158, 1981; Rochefort and Chabos, *Mol. Cell. Endocrinol.* 36: 3-10, 1984; Janne and Bardin, *Pharmacol. Rev.* 36: 35S-42S, 1984; Poyet and Labrie, *Mol. Cell. Endocrinol.* 42: 283-288, 1985; Poulin et al., *Breast Cancer Res. Treatm.* 13: 161-172, 1989). Accordingly, several side effects other than progestational have been noted in patients treated with MPA.

The most easily explained adverse side effects of MPA are related to its glucocorticoid-like action with Cushingoid syndrome, euphoria and subjective pain relief (Mattsson, *Breast Cancer Res. Treatm.* 3: 231-235, 1983; Blosssey et al., *Cancer* 54: 1208-1215, 1984; Hortobagyi et al., *Breast Cancer Res. Treatm.* 5: 321-326, 1985; Van Veelen et al., *Cancer Chemother. Pharmacol.* 15: 167-170, 1985). Suppression of adrenal function by MPA is believed to be caused both by an inhibitory action on ACTH secretion at the pituitary level and by direct inhibition of steroidogenesis at the adrenal level (Blosssey et al., *Cancer* 54: 1208-1215,

1984; Van Veelen et al., *Cancer Chemother. Pharmacol.* 15: 167-170, 1985; Van Veelen et al., *Cancer Treat. Rep.* 69: 977-983, 1985).

Despite its high affinity for AR, MPA seldom causes significant virilizing symptoms (acne, hirsutism, etc.) (Haller and Glick, *Semin. Oncol.* 13: 2-8, 1986). Moreover, its inhibitory effect on gonadotropin secretion is clearly exerted through its direct interaction with pituitary AR in the rat (Labrie et al., *Fertil. Steril.* 28: 1104-1112, 1977; Perez-Palacios et al., *J. Steroid Biochem.* 19: 1729-1735, 1983) and human (Perez-Palacios et al., *J. Steroid Biochem.* 15: 125-130, 1981). In addition, MPA exhibits androgenic activity in the mouse kidney (Janne and Bardin, *Pharmacol. Rev.* 36: 35S-42S, 1980) and in the rat ventral prostate (Labrie, C. et al., *J. Steroid Biochem.* 28: 379-384, 1987; Labrie C. et al., *Mol. Cell. Endocrinol.* 68: 169-179, 1990).

Poulin et al. "Androgen and glucocorticoid receptor-mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells", *Breast Cancer Res. Treatm.* 13: 161-172, 1989) have recently found that the inhibitory effect of medroxyprogesterone acetate (MPA) on the growth of the human ZR-75-1 breast cancer cells is mainly due to the androgenic properties of the compound. The androgenic properties of MPA have been demonstrated in other systems (Labrie C. et al., *J. Steroid Biochem.* 28: 379-384, 1987; Luthy et al., *J. Steroid Biochem.* 31: 845-852, 1988; Plante et al., *J. Steroid Biochem.* 31: 61-64, 1988; Labrie C. et al., *Mol. Cell. Endocrinol.* 58: 169-179, 1990). Other synthetic progestins have also been shown to possess, in addition to their progesterone-like activity, various degrees of androgenic activity (Labrie et al., *Fertil. Steril.* 31: 29-34, 1979; Poyet and Labrie, *The Prostate* 9: 237-246, 1986; Labrie C. et al., *J. Steroid Biochem.* 28: 379-384, 1987; Luthy et al., *J. Steroid Biochem.* 31: 845-852, 1988; Plante et al., *J. Steroid Biochem.* 31: 61-64, 1989).

High dose MPA as first treatment of breast cancer has shown similar effects as Tamoxifen (Van Veelen et al., *Cancer* 58: 7-13, 1986). High dose progestins, especially medroxyprogesterone acetate and megestrol acetate have also been successfully used for the treatment of endometrial cancer (Tatman et al., *Eur. J. Cancer Clin. Oncol.* 25: 1619-1621, 1989; Podratz et al., *Obstet. Gynecol.* 66: 106-110, 1985; Ehrlich et al., *Am. J. Obstet. Gynecol.* 158: 797-807, 1988). High dose MPA is being used with a success similar to that of Tamoxifen for the treatment of endometrial carcinoma (Rendina et al., *Europ. J. Obstet. Gynecol. Reprod. Biol.* 17: 285-291, 1984).

In a randomized clinical trial, high dose MPA administered for 6 months has been shown to induce resolution of the disease in 50% of the patients and a partial resolution in 13% of subjects compared to 12% and 6%, respectively, in patients who received placebo (Telimaa et al., *Gynecol. Endocrinol.* 1: 13, 1987).

The androgen methyltestosterone has been shown to relieve the symptoms of endometriosis (Hamblen, *South Med. J.* 50: 743, 1987; Preston, *Obstet. Gynecol.* 2: 152, 1965). Androgenic and masculinizing side effects (sometimes irreversible) are however important with potent androgenic compounds such as testosterone.

In analogy with the androgen-induced decrease in estrogen receptors in human breast cancer ZR-75-1 cells (Poulin et al., *Endocrinology* 125: 392-399, 1989), oral administration of MPA to women during the follicular phase caused a decrease in the level of estrogen binding in the endometrium (Tseng and Gurbide, *J. Clin. Endocrinol. Metab.* 41: 402-404, 1975).

Studies in animals have shown that androgen deficiency leads to osteopenia while testosterone administration increases the overall quantity of bone (Silberberg and Silberberg, 1971; see Finkelstein et al., *Ann. Int. Med.* 106: 354-361, 1987). Orchiectomy in rats can cause osteoporosis detectable within 2 months (Winks and Felts, *Calcif. Tissue Res.* 32: 77-82, 1980; Verhas et al., *Calif. Tissue Res.* 39: 74-77, 1986).

While hirsute oligomenorrheic and amenorrheic women having low circulating E_2 levels would be expected to have reduced bone mass, these women with high androgen (but low estrogen) levels are at reduced risk of developing osteoporosis (Dixon et al., *Clinical Endocrinology* 30: 271-277, 1989).

Adrenal androgen levels have been found to be reduced in osteoporosis (Nordin et al., *J. Clin. Endocr. Metab.* 60: 651, 1985). Moreover, elevated androgens in postmenopausal women have been shown to protect against accelerated bone loss (Deutsch et al., *Int. J. Gynecol. Obstet.* 25: 217-222, 1987; Aloia et al., *Arch. Int. Med.* 143: 1700-1704, 1983). In agreement with such a role of androgens, urinary levels of androgen metabolites are lower in postmenopausal symptomatic menopause than in matched controls and a significant decrease in conjugated dehydroepiandrosterone (DHEA) was found in the plasma of osteoporotic patients (Hollo and Feher, *Acta Med. Hung.* 20: 133, 1964; Urist and Vincent, *J. Clin. Orthop.* 18: 199, 1961; Hollo et al., *Acta Med. Hung.* 27: 155, 1970). It has even been suggested that postmenopausal osteoporosis results from both hypoestrogenism and hypoandrogenism (Hollo et al., *Lancet*: 1357, 1976).

As a mechanism for the above-suggested role of both estrogens and androgens in osteoporosis, the presence of estrogen (Komm et al., *Science* 241: 81-84, 1988; Eriksen et al., *Science* 241: 84-86, 1988) as well as androgen (Colvard et al., *Proc. Natl. Acad. Sci.* 86: 854-857, 1989) receptors in osteoblasts could explain increased bone resorption observed after estrogen and androgen depletion.

In boys, during normal puberty, an increase in serum testosterone levels precedes an increase in alkaline phosphatase activity (marker of osteoblastic activity) which itself precedes increased bone density (Krabbe et al., *Arch. Dis. Child.* 54: 950-953, 1979; Krabbe et al., *Arch. Pediat. Scand.* 73: 750-755, 1984; Riis et al., *Calif. Tissue Res.* 37: 213-217, 1985).

While, in women, there is a rapid bone loss starting at menopause, bone loss in males can be recognized at about 65 years of age (Riggs et al., *J. Clin. Invest.* 67: 328-335, 1987). A significant bone loss is seen in men at about 80 years of age, with the accompanying occurrence of hip, spine and wrist fractures. Several studies indicate that osteoporosis is a clinical manifestation of androgen deficiency in men (Baran et al., *Calcif. Tissue Res.* 26: 103-106, 1978; Odell and Swerdloff, *West. J. Med.* 124: 446-475, 1976; Smith and Walker, *Calif. Tissue Res.* 22 (Suppl.): 225-228, 1976).

Although less frequent than in women osteoporosis can cause significant morbidity in men (Seeman et al., *Am. J. Med.* 75: 977-983, 1983). In fact, androgen deficiency is a major risk for spinal compression in men (Seeman et al., *Am. J. Med.* 75: 977-983, 1983). Decreased radial and spinal bone density accompanies hypogonadism associated with hyperprolactinemia (Greenspan et al., *Ann. Int. Med.* 104: 777-782, 1986) or anorexia nervosa (Rigotti et al., *JAMA* 256: 385-288, 1986). However, in these cases, the role of hyperprolactinemia and loss in body weight is uncertain.

Hypogonadism in the male is a well-recognized cause of osteoporotic fracture (Albright and Reifstein, 1948; Saville, *Clin. End. Metab.* 2: 177-185, 1973). Bone density is in fact reduced in both primary and secondary hypogonadism (Velentzas and Karras, *Nouv. Presse Médicale* 10: 2520, 1981).

Severe osteopenia as revealed by decreased cortical and trabecular bone density was reported in 23 hypogonadotropic hypogonadal men (Finkelstein et al., *Ann. Int. Med.* 106: 354-361, 1987; Foresta et al., *Horm. Metab. Res.* 15: 56-57, 1983). Osteopenia has also been reported in men with Klinefelter's syndrome (Foresta et al., *Horm. Metab. Res.* 15: 206-207, 1983; Foresta et al., *Horm. Metab. Res.* 15: 56-57, 1983; Smith and Walker, *Calif. Tissue Res.* 22: 225-228, 1977).

Androgenic-reversible decreased sensitivity to calcitonin has been described in rats after castration (Ogata et al., *Endocrinology* 87: 421, 1970; Hollo et al., *Lancet* 1: 1205, 1971; Hollo et al., *Lancet* 1: 1357, 1976). In addition, serum calcitonin has been found to be reduced in hypogonadal men (Foresta et al., *Horm. Metab. Res.* 15: 206-207, 1983) and testosterone therapy in castrated rats increases the hypocalcemic effect of calcitonin (McDermatt and Kidd, *End. Rev.* 8: 377-390, 1987).

Albright and Ruferstein (1948) originally suggested that androgens increase the synthesis of bone matrix. Androgens have also been shown to increase osteoid synthesis and mineralization in chicken (Puche and Rosmano, *Calif. Tissue Res.* 4: 39-47, 1969). Androgen therapy in hypogonadal men increases skeletal growth and maturation (Webster and Hogkins, *Proc. Soc. Exp. Biol. Med.* 45: 72-75, 1940). In addition, testosterone therapy in man has been shown to cause positive nitrogen, calcium and phosphate balance (Albright, F., Reifeinstein, E. C. In: *The parathyroid glands and metabolic bone disease*. Williams and Williams Co.: Baltimore, pp. 145-204, 1948). As studied by bone histomorphometry, testosterone therapy in hypogonadal males resulted in increases in relative osteoid volume, total osteoid surface, linear extend of bone formation and bone mineralization (Barau et al., *Calcif. Tissue Res.* 26: 103-106, 1978).

Treatment with testosterone has been shown to increase osteoid surfaces and beam width with unchanged or reduced oppositional rates, thus indicating and increase in total bone mineralization rate (Peacock et al., *Bone* 7: 261-268, 1986). There was also a decrease in plasma phosphate probably due to an effect on renal tubular reabsorption of phosphates (Selby et al., *Clin. Sci.* 69: 265-271, 1985).

Cortical bone density increases in hyperprolactinemic men with hypogonadism when testicular function is normalized (Greenspan et al., *Ann. Int. Med.* 104: 777-782, 1986; Greenspan et al., *Ann. Int. Med.* 110: 526-531, 1989). Testosterone therapy increases bone formation in men with primary hypogonadism (Baron et al., *Calcif. Tissue Res.* 26: 103-106, 1978; Francis et al., *Bone* 7: 261-268, 1986).

In 21 hypogonadal men with isolated GnRH deficiency, normalization of serum testosterone for more than 12 months increased bone density (Kinkelstein et al., *J. Clin. Endocr. Metab.* 69: 776-783, 1989). In men with already fused epiphyses, however, there was a significant increase in cortical bone density while no significant change was observed on trabecular bone density, thus supporting previous suggestions of variable sensitivity of cortical and trabecular bone to steroid therapy.

Previous studies with anabolic steroids in small numbers of patients have suggested positive effects on bone (Lafferty

et al., *Ann. J. Med.* 36: 514-528, 1964; Riggs et al., *J. Clin. Invest.* 51: 2659-2663, 1972; Harrison et al., *Metabolism* 20: 1107-1118, 1971). More recently, using total body calcium measurements by neutron activation as parameter, the anabolic steroid methandrostenolone has shown positive and relatively long-term (24-26 months) effects in a double-blind study in postmenopausal osteoporosis (Chessnut et al., *Metabolism* 26: 267-277, 1977; Aloia et al., *Metabolism* 30: 1076-1079, 1981).

The anabolic steroid nandrolone decanoate reduced bone resorption in osteoporotic women (Dequeker and Geusens, *Acta Endocrinol.* 271 (Suppl.): 45-52, 1985) in agreement with the results observed during estrogen therapy (Dequeker and Ferin, 1976, see Dequeker and Geusens). Such data confirm experimental data in rabbits and dogs when nandrolone decanoate reduced bone resorption (Ohem et al., *Curr. Med. Res. Opin.* 6: 606-613, 1980). Moreover, in osteoporotic women (Dequeker and Geusens, *Acta Endocrinol.* (Suppl.) 271: 45-52, 1985) the anabolic steroid not only reduced bone loss but also increased bone mass. Vitamin D treatment, on the other hand, only reduced bone resorption.

Therapy of postmenopausal women with nandrolone increased cortical bone mineral content (*Clin. Orthop.* 225: 273-277). Androgenic side effects, however, were recorded in 50% of patients. Such data are of interest since while most therapies are limited to an arrest of bone loss, an increased in bone mass was found with the use of the anabolic steroid nandrolone. A similar stimulation of bone formation by androgens has been suggested in a hypogonadal male (Baran et al., *Calcif. Tissue Res.* 26: 103, 1978). The problem with regimens which inhibit bone resorption with calcium, calcitriol or hormones is that they almost certainly lead to suppression of bone formation (Need et al., *Mineral. Electrolyte Metabolism* 11: 35, 1985). Although, Albright and Reiferestein (1948) (See Need, *Clin. Orthop.* 225: 273, 1987) suggested that osteoporosis is related to decreased bone formation and will respond to testosterone therapy, the virilizing effects of androgens have made them unsuitable for the treatment of postmenopausal women. Anabolic steroids, compounds having fewer virilizing effects, were subsequently developed. Although, minimal effects have been reported by some (Wilson and Griffin, *Metabolism* 28: 1278, 1980) more positive results have been reported (Chessnut et al., *Metabolism* 32: 571-580, 1983; Chessnut et al., *Metabolism* 26: 267, 1988; Dequeker and Geusens, *Acta Endocrinol.* (Suppl. 110) 271: 452, 1985). A randomized study in postmenopausal women has been shown an increase in total bone mass during treatment with the anabolic steroid stanazolol although side effects were recorded in the majority of patients (Chessnut et al., *Metabolism* 32: 571-580, 1983).

As mentioned above, the doses of "progestins" (for example medroxyprogesterone acetate) used for the standard therapy of breast cancer are accompanied by undesirable important side effects (especially those related to interaction of the steroid with the glucocorticoid receptor, especially Cushingoid syndrome, euphoria) (Mattsson, *Breast Cancer Res. Treatm.* 3: 231-235, 1983; Blosssey et al., *Cancer* 54: 1208-1215, 1984; Hortobagyi et al., *Breast Cancer Res. Treatm.* 5: 321-326, 1985; Von Veelen et al., *Cancer Chemother. Pharmacol.* 15: 167-170, 1985).

The term "progestin" refers to derivatives of progesterone and testosterone. Such progestins have, at times, been synthesized with the aim of developing compounds acting as analogs of progesterone on the progesterone receptors, especially for the control of fertility. With the availability of new

and more precise tests, however, it became evident that such compounds, originally made to interact exclusively with the progesterone receptor, do also interact, frequently with high affinity, with the androgen receptor (Labrie et al., *Fertil. Steril.* 28: 1104-1112, 1977; Labrie et al., *Fertil. Steril.* 31: 29-34, 1979; Labrie, C. et al., *J. Steroid Biochem.* 28: 379-384, 1987; Labrie C. et al., *Mol. Cell. Endocrinol.* 68: 169-179, 1990). Sometimes, the androgenic activity of these compounds, especially at low concentrations, becomes more important than the true progestin activity. This is the case, for example, for medroxyprogesterone acetate (Poulin et al., *Breast Cancer Res. Treatm.* 13: 161-172, 1989).

A problem with prior-art treatments of breast and endometrial cancer with synthetic progestins is the side effects observed with such treatments. The blockade of estrogens, another common treatment for breast cancer, would have undesirable deleterious effects on bone mass in women. Similarly, blockade of estrogens, a common treatment for endometriosis, has similar undesirable deleterious effects on bone mass in women.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for prevention and treatment of breast cancer, endometrial cancer, osteoporosis and endometriosis, while substantially avoiding undesirable side effects.

It is another object of the invention to provide a method for prevention of cancer having more specific effectiveness in delaying tumor growth.

It is another object of the invention to provide a method for prevention of breast and endometrial cancer having significantly reduced frequency of unwanted side effects.

It is another object of the invention to provide a method for prevention of bone loss in men and women having a reduced frequency of unwanted side effects.

It is another object of the invention to provide a method for prevention of bone loss in women where estrogen formation and/or action is blocked in order to treat various estrogen-sensitive diseases, including cancer.

It is another object of the invention to provide a method for prevention of bone loss in women already exposed to low estrogens following menopause.

It is a further object of the invention to provide kits and pharmaceutical compositions for use in the methods described herein.

These and other objects are achieved by practicing the methods disclosed herein and/or by utilizing the pharmaceutical compositions and kits disclosed herein.

In one embodiment, a method is provided for activating androgen receptors in a warm blooded animal, including a human, comprising administering to said animal at least one androgenic steroid having a K_i value of less than $2 \times 10^{-8} M$ for the androgen receptor, an androgen receptor-mediated inhibitory effect on the growth of human breast cancer ZR-75-1 cells which reaches half-maximal value at a concentration below 3.0 nanomoles per liter, and no visible masculinizing activity at blood serum concentrations below 50 nM, wherein every such androgenic steroid is administered at a dosage sufficiently low to maintain a cumulative serum concentration below 50 nanomoles per liter.

The methods of said androgenic steroid described herein are particularly useful for the treatment of human breast or endometrial cancer, osteoporosis or endometriosis. It is believed that the methods are also suitable for all purposes which are enhanced by administering androgens or other-

wise activating androgen receptors. Both treatment and prevention of the diseases and disorders discussed herein are contemplated within the scope of the invention. It is believed that the methods of the invention are suitable for both prophylactic and therapeutic use.

The compounds utilized have the special property of possessing potent androgenic activity at low blood concentration (e.g. less than 50 nM) while exhibiting very little glucocorticoid receptor activity at those concentrations. They are also characterized by the absence of physical masculinizing activity in females at the concentration range at which they are used. This is to be distinguished from natural androgens produced in gonadal or peripheral tissues such as testosterone and dihydrotestosterone which exhibit considerable masculinizing activity even at low blood concentrations. Synthetic progestins, e.g. progesterone derivatives are useful for this invention, as are some anabolic steroids.

The androgens of the invention on average do not cause physically detectable increase in masculinizing effects such as increased hair growth in females, acne, seborrhea or hair loss. These masculinizing effects have been quantified in the literature. See, for example, Ferriman and Gallwey, J. P. Clin. Endocrinol. Metab. 21: 1440-1447, 1961 (regarding hair growth); Cremoncini et al., Acta. Eur. Fert. 7: 248-314, 1976 (acne, seborrhea and hair loss). See also Cusan et al., J. Am. Acad. Dermatol. 23: 462-469, 1990. Tables 1 and 2 below set forth a quantification.

TABLE 1

Definition of hair grading at each of 11 sites (Grade 0 at all sites indicates absence of terminal hair)		
Site	Grade	Definition
1. Upper lip	1	A few hairs at outer margin
	2	A small moustache at outer margin
	3	A moustache extending halfway from outer margin
2. Chin	4	A moustache extending to mid-line
	1	A few scattered hairs
3. Chest	2	Scattered hairs with small concentrations
	3 & 4	Complete cover, light and heavy
	1	Circumareolar hairs
4. Upper back	2	With mid-line hair in addition
	3	Fusion of these areas, with three-quarter cover
	4	Complete cover
5. Lower back	1	A few scattered hairs
	2	Rather more, still scattered
	3 & 4	Complete cover, light and heavy
6. Upper abdomen	1	A sacral tuft of hair
	2	With some lateral extension
	3	Three-quarter cover
7. Lower abdomen	4	Complete cover
	1	A few mid-line hairs
	2	Rather more, still mid-line
8. Arm	3 & 4	Half and full cover
	1	A few mid-line hairs
	2	A mid-line streak of hair
9. Forearm	3	A mid-line band of hair
	4	An inverted V-shaped growth
	1	Sparse growth affecting not more than a quarter of the limb surface
10. Thigh	2	More than this; cover still incomplete
	3 & 4	Complete cover, light and heavy
	1, 2, 3, 4	Complete cover of dorsal surface; 2 grades of light and 2 of heavy growth
11. Leg	1, 2, 3, 4	As for arm
	1, 2, 3, 4	As for arm

TABLE 2

Grading of Acne, Seborrhea and Hair Loss	
<u>Acne</u>	
1.	Isolated pustules, up to 10 in number
2.	More than 10 isolated pustules
3.	Clusters of pustules
4.	Confluent pustules
<u>Seborrhea</u>	
1.	Mild
2.	Moderate
3.	Severe
<u>Hair Loss</u>	
1.	Mild
2.	Obvious thinning
3.	Very obvious thinning
4.	Baldness

Preferred compounds for use in the invention include synthetic progestins, anabolic steroids and other steroidal compounds having a K_i value of less than $2 \times 10^{-8} M$ for the androgen receptor, an androgen receptor-mediated inhibitory effect on the growth of human breast cancer ZR-75-1 cells reaching half-maximal value at a concentration below 3.0 nanomoles per liter, and lacking the masculinizing activity discussed above. Preferred androgens of the invention would cause no significant increase in the average masculinizing effect (e.g. a significant increase in any of the numerical grades set forth in Tables 1 or 2 above) observed in females following treatment for three months with blood concentrations of the androgen maintained at the top of the claimed concentration range (e.g. 50 nanomoles per liter). For most female patients for whom no masculinizing effects were visible prior to treatment, or a total score of 10 or less for all 11 sites indicated in Table 1 prior to treatment, the same score would normally be maintained during treatment in accordance with the present invention. That is, there would be no visible masculinizing effects after three months of treatment. For female patients displaying some masculinizing effects prior to treatment, it would be expected that those effects would not be increased by treatment.

To determine whether the K_i values are below $2 \times 10^{-8} M$, K_i values may be determined by the following method for measuring the affinity of various compounds for the androgen receptor.

Preparation of prostatic tissue

Ventral prostates are from Sprague-Dawley rats (CrI:CD (SD)Br) (obtained from Charles River, St-Constant, Québec) weighing 200-250 g and castrated 24 h before sacrifice. Immediately after removal, prostates are kept on ice and used for the androgen binding assays.

Preparation of cytosol

Prostatic tissues are finely minced with scissors (fresh tissue) or pulverized with a Thermovac system (frozen tissue) before homogenization in buffer A (Tris, 0.025M; monothioglycerol, 20 mM; glycerol, 10% (v/v); EDTA, 1.5 mM and sodium molybdate, 10 mM, pH 7.4) in a 1:5 ratio (w/v) using a Polytron PT-10 homogenizer. These and all the following procedures are performed at $0^\circ-4^\circ C$. The homogenate is centrifuged at $105000 \times g$ for 1 h in order to obtain the cytosolic fraction in the supernatant.

Cytosolic androgen receptor assay

Aliquots of $100 \mu l$ are incubated at $0^\circ-4^\circ C$. for 18 h with $100 \mu l$ of 3 nM [3H] T or [3H] R1881 in the presence or

absence of increasing concentrations of the non-labeled androgenic compound to be tested. At the end of the incubation, free and bound T or R1881 are separated by the addition of 200 μ l dextran-coated charcoal (1% charcoal, 0.1% dextran T-70, 0.1% gelatin, 1.5 mM EDTA and 50 mM Tris (pH 7.4)) for 15 min before centrifugation at 2300xg for another 15 min at 0°-4° C. Aliquots (350 μ l) of the supernatant are transferred to scintillation vials with 10 ml of an aqueous counting solution (Formula 963, New England Nuclear) before counting in a Beckman LS 330 counter (30% efficiency for tritium).

Ki calculation

Apparent inhibition constant "Ki" values are calculated according to the equation $Ki = IC_{50} / (1 + S/K)$ (Cheng and Prusoff, *Biochem. Pharmacol.* 22: 3099-3108, 1973). In this equation, S represents the concentration of [³H]T or [³H] R1881, K is the dissociation constant (K_D) of T or R1881 and IC_{50} is the concentration of unlabeled compounds giving a 50% inhibition of T or R1881 binding. For numerous compounds, Ki values are reported in the literature. See, for example, Ojasso et al., *J. Ster. Biochem.* 27: 255-269, 1987; Asselin et al., *Cancer Res.* 40: 1612-1622, 1980; Toth and Zakar J. *Steroid Biochem.* 17: 653-660, 1982. A method giving similar results is described in Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988.

In order to determine the concentration at which a given compound reaches half-maximal androgen receptor-mediated inhibitory effect on the growth of human breast cancer ZR-75-1 cells, the following technique is utilized as described in detail in Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988.

Maintenance of stock cultures

The ZR-75-1 human breast cancer cell line can be obtained from the American Type Culture Collection (Rockville, Md.). The cells are routinely cultured in phenol red-free RPMI 1640 medium supplemented with 10 nM E_2 , 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin per ml, 100 μ g streptomycin sulfate per ml, and 10% (v/v) fetal bovine serum (FBS), in a water-saturated atmosphere of 95% air: 5% CO_2 at 37° C.

Stock cultures in their logarithmic growth phase are harvested with 0.05% trypsin/0.02% EDTA (w/v) in Hanks' balanced salts solution and resuspended in E_2 - and phenol red-free RPMI 1640 medium containing 5% (v/v) dextran-coated charcoal (DCC)-treated FBS and 500 ng of bovine insulin per ml, but otherwise supplemented as described above for maintenance of stock cultures. Cells were plated in 24-well Linbro culture plates (Flow Laboratories) at a final density of $0.5-4.0 \times 10^4$ cells/well.

Forty-eight hours after plating, fresh SD medium containing the appropriate concentrations of steroids are added. The final concentration of ethanol used for the addition of test substances does not exceed 0.12% (v/v) and has no significant effect on cell growth and morphology. The incubation media are replaced every other day and cells are harvested by trypsinization after 12 days of treatment, unless otherwise indicated. Cell number can be determined with a Coulter Counter.

Calculations and statistical analyses

Apparent IC_{50} values are calculated using an iterative least squares regression (Rodbard, *Endocrinology* 94: 1427-1437, 1974), while apparent inhibition constants (Ki values) are calculated according to Cheng and Prusoff (*Biochem. Pharmacol.* 22: 3099-3108, 1973).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a comparative graph over time of the number of tumors observed in a group of rats protected by a method in

accordance with the invention following administration of dimethylbenz(a)anthracene (DMBA) versus an unprotected control group.

FIG. 2 is a comparative graph of estradiol-stimulated growth of tumors in ovariectomized rats treated in accordance with the invention versus an untreated control group.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A better understanding of the multiple endocrine activity of synthetic progestins is required not only for their more rational use in the prevention and therapy of breast and endometrial cancers as well as endometriosis and bone loss but also to avoid side effects caused by interaction with steroid receptors unnecessary for the desired beneficial effect.

Precise analysis of the biological actions of synthetic "progestins" having affinity for many steroidal receptors would ideally require the selection of in vitro models possessing functional receptors for all major classes of steroids. For this purpose, we have chosen the ZR-75-1 human breast cancer cell line, which possesses functional receptors for estrogens, androgens, progesterone and glucocorticoids (Vignon et al., *J. Clin. Endocrinol. Metab.* 56: 1124-1130, 1983) in order to compare the relative contribution of the different steroid receptor systems in the control of cell proliferation by synthetic progestins. While estrogens are strongly mitogenic in ZR-75-1 cells (Poulin and Labrie, *Cancer Res.* 46: 4933-4937, 1986) and specifically regulate the expression and/or the secretion of several proteins (Dickson and Lippman, *Endocr. Rev.* 8: 29-43, 1987), androgens (Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988), glucocorticoids (Hatton, A. C., Labrie, F., unpublished results) as well as progestins (Poulin et al., *Breast Cancer Res. Treatm.* 13: 161-172, 1989) inhibit their proliferation through specific interactions with their respective receptors.

Many progestins have been used in the treatment of breast cancer, including MPA (Blossey et al., *Cancer* 54: 1208-1215, 1984; Hortobayyi et al., *Breast Cancer Res. Treatm.* 5: 321-326, 1985), MGA (Johnson et al., *Semin. Oncol.* 13 (Suppl.): 15-19, 1986; Tchekmedyan et al., *Semin. Oncol.* 13 (Suppl.): 20-25, 1986) and norethindrone (Clavel et al., *Eur. J. Cancer Clin. Oncol.* 18: 821-826, 1982; Earl et al., *Clin. Oncol.* 10: 103-109, 1984). Using the in vitro system of human breast cancer ZR-75-1 cells, I have found that the synthetic progestins or anabolic steroids, Nor-testosterone, R1881, dromostanolone, fluoxymesterone, ethisterone, methandrostanolone, oxandrolone, danazol, stanozolol, calusterone, oxymetholone, cyproterone acetate, chlormadinone acetate and norgestrel, possess androgenic activity at low concentrations. In addition to inhibition of cell growth, the secretion of two glycoproteins, namely gross cystic disease fluid protein-15 (GCDFP-15) and GCDFP-24 is markedly stimulated by androgens (Simard et al., *Mol. Endocrinol.* 3: 694-702, 1989; Simard et al., *Endocrinology* 126: 3223-3231, 1990). Measurements of GCDFP-25 or GCDFP-24 secretion can thus be used as sensitive parameter or marker of androgen action in these cells. In fact, changes in GCDFP-15 and GCDFP-24 secretion are opposite to the changes in cell growth under all experimental conditions examined. All the synthetic progestins or anabolic steroids that I have studied exhibit androgenic activity on ZR-75-1 breast cancer growth and secretion of GCDFP-15 and GCDFP-24.

Identification of the receptors (estrogen, androgen, progesterone and glucocorticoid) responsible for the action of the compounds is essential in order to assess the potential actions (including adverse effects) of such compounds. It is thus especially important to assess the specific interaction at low concentrations with the androgen receptor since such low concentrations do not interact with the glucocorticoid receptor, thus avoiding or minimizing secondary side effects.

One method for inhibiting growth of breast and endometrial cells is activation of the androgen receptor with an effective compound having an affinity for the receptor site such that it binds to the androgen receptor at low concentrations while not significantly activating other classes of steroid receptors linked to potential side effects. It is important to select compounds having maximal affinity for the androgen receptor which have minimal or no virilizing effects in women. In order to minimize interaction of such compounds with the glucocorticoid and estrogen receptors, it is important to use low dose of the compounds. It is also important to choose steroids having androgenic activity at low concentrations which are not metabolized into estrogens under in vivo conditions which, at the low concentrations used, will not lead to significant activation of receptors other than the androgen receptors.

My research has shown that the compounds used in the invention, particularly anabolic steroids and synthetic progestins, vary markedly, over different concentrations, in their ability to activate different classes of steroidal receptors. Hence, by carefully controlling concentration, it is possible to selectively cause activation of desired receptors while not causing significant activation of undesired receptors. For example, at the low concentrations specified herein, MPA can be utilized to desirably activate androgen receptors while substantially avoiding side effects associated with glucocorticoid activation which have plagued prior art treatments.

Thus, this invention provides a novel method for prevention and therapy of breast and endometrial cancer as well as other diseases responsive to activation of the androgen receptor, e.g. bone loss and endometriosis. In this invention, the amount of the androgenic compounds administered is much lower than previously used in art for the treatment of breast and endometrial cancer.

MONITORING BLOOD CONCENTRATION OF ANDROGENS OF THE INVENTION

To help in determining the potential effects of the treatment, blood concentrations of the compound can be measured. For example, measurements of plasma medroxyprogesterone acetate (MPA) levels can be made by radioimmunoassay following extraction as follows:

Antibody preparation

Antibody 144A was raised in rabbits against 17-hydroxyprogesterone-3-O-carboxymethylloxime-BSA. The labeled steroid used in the radioimmunoassay (RIA) was methyl-17 α -hydroxyprogesterone acetate, 6 α -[1,2-³H (N)]- obtained from NEN (CAT NO: NET 480) while the reference preparation was medroxyprogesterone acetate (MPA) obtained from Steraloids. The assay buffer used was 0.1% gelatin in 0.1M sodium phosphate, 0.15M sodium chloride, 0.1% sodium azide, pH 7.2. The extraction solvent mixture was ethyl ether-acetone (9:1, v:v) [EEA] while the LH-20 chromatography solvent mixture was iso-octane: toluene: methanol (90:5:5; v:v:v) [IOTH].

Extraction procedure

One ml of plasma was extracted twice with 5 ml of EEA. The extracts were evaporated to dryness with nitrogen and

the remaining residue was dissolved in one ml of IOTH. The extracts were then subjected to LH-20 chromatography on 10x30 cm columns (Corning CAT NO: 05 722A) filled with 2 g of LH-20 (Pharmacia). The gel was washed with 30 ml of IOTH before addition of one ml of sample and elution with IOTH. The first 6 ml were discarded. The following 10, 16.5 and 27.5 ml of eluent were fraction I (progesterone), II (MPA) and III (17-LH-progesterone), respectively. Fraction II was evaporated to dryness and reconstituted in 1.5 ml of assay buffer.

Radioimmunoassay

To each 12x75 mm borosilicate test tube was added: 0.2 ml containing 25,000 DPM of tritiated steroid, 0.5 ml of reference preparation ranging from 5 to 5000 pg/tube or 0.5 ml of extracted sample fraction II, 0.2 ml of antiserum 144A diluted 1/5000 or 0.2 ml of assay buffer to account for non specific binding. The tubes were then incubated overnight at 4° C. Then, 0.2 ml 2% charcoal Norit-A, 0.2% Dextran T-70 diluted in water was added. The tubes were then shaken gently and, after 10 min, they were centrifuged at 2000xg for 10 min. The supernatant was mixed with 8 ml of Formula-989 (NEN: NEF-989) and the radioactivity was counted in a β -counter.

The lower and upper limits of detection of MPA are 10 and 10000 pg/ml, respectively, while the slope (LOGIT-LOG) is -2.2 and the ED₅₀ value is 315 pg/ml. Non-specific and net binding are 1.5 and 45%, respectively. Antibody 144A is highly specific for MPA since cross-reactivity with progesterone, 20 α -OH-Prog, pregnenolone, 17-OH-pregnenolone, DHT, androstenedione, testosterone, 3 α -diol, estrone, estradiol and cortisol is less than 0.1%.

Calculations and statistics

RIA data were analyzed using a program based on model II of Roadbard and Lewald (In: 2nd Karolinska Symposium, Geneva, 1970, pp. 79-103). Plasma MPA levels are usually shown as the means \pm SEM (standard error of the mean) of duplicate measurements of individual samples. Statistical significance is measured according to the multiple-range test of Duncan-Kramer (Kramer, C. Y., Biometrics 12: 307-310, 1956).

A test compound's relative effect on various receptors

To assist in determining the activity of the potential compounds on the various steroid receptors, androgen, glucocorticoid, progesterone and estrogen-receptor-mediated activities of synthetic progestins and anabolic steroids can be measured in ZR-75-1 human breast cancer cells using cell growth as well as GCDFP-15 and GCDFP-24 release as parameters of response (Poulin and Labrie, Cancer Res. 46: 4933-4937, 1986; Poulin et al., Breast Cancer Res. Treatm. 12: 213-225, 1988; Poulin et al., Breast Cancer Res. Treatm. 13: 161-172, 1989; Poulin et al., Breast Cancer Res. Treatm. 13: 265-276, 1989; Simard et al., Mol. Endocrinol. 3: 694-702, 1989; Simard et al., Endocrinology 126: 3223-3231, 1990).

The following properties permit measurement of progesterone receptor (PgR) activity: 1) the addition of insulin completely reverses the inhibition due to the interaction of the progestin R5020 with the PgR in ZR-75-1 cells; and 2) the antiproliferative effect of R5020 is observed only under E₁-stimulated conditions. These two characteristics of ZR-75-1 cell growth permit study of the extent to which a tested compound's effects on ZR-75-1 cells are attributed to its interaction with PgR by evaluating the effect of insulin and/or estrogen addition on the growth response measured at the end of a 15-day incubation of ZR-75-1 cells with the test compounds.

The contribution of the estrogen receptor (ER), on the other hand, can be directly measured by incubating ZR-75-1 cells in the presence or absence of estrogen in the medium.

In order to analyze the interactions of synthetic progestins or anabolic steroids with the androgen receptor (AR) and glucocorticoid receptor (GR) in their inhibitory action on cell growth, one takes advantage of the additivity of the anti-proliferative effects of androgens and glucocorticoids in this cell line (Poulin et al., *Breast Cancer Res. Treatm.* 12: 213-225, 1988; Hatton and Labrie, F., unpublished data). Thus, one can saturate AR with 5 α -dihydrotestosterone (DHT) and then measure the effect on cell proliferation resulting from the addition of a putative glucocorticoid. On the other hand, the effect of a putative androgen can similarly be measured following saturation of GR by dexamethasone (DEX). The specificity of the growth-inhibitory activity thus observed with the test compound can also be further assessed by its reversibility using the appropriate antagonist (i.e. antiglucocorticoid or antiandrogen). Thus, in the presence of excess androgen (1 μ M DHT) in the presence of E₂ and insulin, glucocorticoid effects can be assessed with precision and with no interference by the other receptors. The same applies to study of the role of AR when the cells are incubated in the presence of excess glucocorticoid (3 μ M DEX), in the presence of E₂ and insulin. As demonstrated by detailed kinetic studies, 1 μ M DHT and 3 μ M DEX exert maximal inhibitory effects on the AR and GR, respectively.

In addition, the possible antagonistic activities of "progestins" mediated through the AR and GR can be determined by saturating both receptor systems with DHT and DEX with one ligand being in far greater excess than the other in order to allow reversal through a single chosen receptor at a time. All experiments are performed with ZR-75-1 cells grown in E₂-supplemented media containing insulin in order to prevent the PgR-mediated effect of "progestins" on cell growth.

Using the foregoing techniques, I have found that numerous androgenic compounds which also activate other receptors (e.g. glucocorticoid or progesterone receptors) vary in their relative effects on different receptors as a function of concentration. By staying within concentration ranges defined herein, compounds of the invention may beneficially affect androgen receptors without substantial undesirable effects on other receptors.

Selection of patients who may benefit from the method's described herein

The appearance of breast cancer is usually detected by self breast examination and/or mammography. Endometrial cancer, on the other hand, is usually diagnosed by endometrial biopsy. Both cancers can be diagnosed and evaluated by standard physical methods well known to those skilled in the art, e.g. bone scan, chest X-Ray, skeletal survey, ultrasonography of the liver and liver scan (if needed), CAT scan, MRI and physical examination. Endometriosis can be diagnosed following pains or symptoms associated with menstruations in women while definitive diagnosis can be obtained by laparoscopy and, sometimes, biopsy.

Bone density, on the other hand, can be measured by standard methods well known to those skilled in the art, e.g. QDR (Quantitative Digital Radiography), dual photon absorptiometry and computerized tomography. Plasma and urinary calcium and phosphate levels, plasma alkaline phosphatase, calcitonin and parathormone concentrations, as well as urinary hydroxyproline and calcium/creatinine ratios.

Breast or endometrial cancer, osteoporosis or otherwise insufficient bone mass, and other diseases treatable by activating androgen receptor may be treated in accordance with the present invention or prophylactically prevented in accordance herewith.

Typically suitable androgenic compounds include 6-alpha-methyl,17-alpha-acetoxy progesterone or medroxyprogesterone acetate available, for example, from Upjohn and Farmitalia Carlo Erba, S.p.A. under the trade names Provera, DepoProvera or Farlutal, and the acronym MPA.

Other suitable androgenic compounds include those described in Labrie et al. (*Fertil. Steril.* 31: 29-34, 1979) as well as anabolic steroids or progestins (Raynaud and Ojasso, In: *Innovative Approaches in Drug Research*, Elsevier Sci. Publishers, Amsterdam, pp. 47-72, 1986; Sandberg and Kirdoni, *Pharmac. Ther.* 36: 263-307, 1988; and Vincens, Simard and De Lignières, *Les Androgènes*. In: *Pharmacologie Clinique, Base de Thérapeutique*, 2ième Edition, Expansion Scientifique (Paris), pp. 2139-2158, 1988), as well as Calusterone (7 β ,17 α -dimethyltestosterone), anabolic steroids (Lam, *Am. J. Sports Medicine* 12, 31-38, 1984; Hilf, R., *Anabolic-androgenic steroids and experimental tumors*. In: (Kochachian, C. D., eds.), *Handbook of Experimental Pharmacology*, vol. 43, *Anabolic-Androgenic Steroids*, Springer-Verlag, Berlin, 725 pp, 1976), fluoxymesterone (9 α -fluoro-11 β -hydroxy-17 α -methyltestosterone), testosterone 17 β -cypionate, 17 α -methyltestosterone, Pantestone (testosterone undecanoate), Δ^1 -testololactone and Andrac-tim.

Other typical suitable androgenic compounds are cyproterone acetate (Androcur) available from Shering AG, 6-alpha-methyl, 17-alpha-acetoxy progesterone or medroxyprogesterone acetate (MPA) available from, among others, Upjohn and Farmitalia, Calbo ERba, Gestodene available from Shering, megestrol acetate (17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione) available from Mead Johnson & Co., Evansville, Ind., under the trade name of Megace. Other synthetic progestins include Levonorgestrel, Norgestimate, desogestrel, 3-ketodesogestrel, norethindrone, norethisterone, 13 α -ethyl-17-hydroxy-18, 19-dinor-17 β -pregna-4,9,11-triene-20-yn-3-one (R2323, gestrinone), demegestone, norgestrienone, gastrinone and others described in Raynaud and Ojasso, *J. Steroid Biochem.* 25: 811-833, 1986; Raynaud et al., *J. Steroid Biochem.* 25: 811-833, 1986; Raynaud et al, *J. Steroid Biochem.* 12: 143-157, 1980; Raynaud, Ojasoo and Labrie, *Steroid Hormones, Agonists and Antagonists*, In: *Mechanisms of Steroid Action* (G. P. Lewis and M. Ginsburg, eds), McMillan Press, London pp. 145-158 (1981). Any other progestin derivative having the above-described characteristics could also be useful for the invention.

The androgenic compound is preferably administered as a pharmaceutical composition via topical, parenteral or oral means. The compound can be administered parenterally, i.e. intramuscularly or subcutaneously by injection of infusion by nasal drops, by suppository, or where applicable intravaginally or transdermally using a gel, a patch or other suitable means. The androgenic compound may also be microencapsulated in or attached to a biocompatible, biodegradable polymer, e.g. poly(d,l-lactide-co-glycolide) and subcutaneously or intramuscularly injected by a technique called subcutaneous or intramuscular depot to provide continuous, slow release of the compound over a period of 30 days or longer. In addition to the oral route, a preferred route of administration of the compound is subcutaneous depot injection. DepoProvera can be released at a relatively constant rate for approximately 3 months after intramuscular administration of an aqueous suppression.

The amount of each compound administered is determined by the attending clinician taking into consideration the patient's condition and age, the potency of each component and other factors. In the prevention of breast and

endometrial cancer, as well as bone loss, according to this invention, the following dosage ranges are suitable.

The androgenic composition is preferably administered in a daily dosage which delivers less than 25 mg of active androgenic steroid per 50 kg of body weight.

A dosage of 1–10 mg per 50 kg of body weight, especially 3–7 mg (e.g. 5 mg) is preferred. The dosage selected preferably maintains serum concentration below 50 nanomoles per liter, preferably between 1.0 nanomoles per liter and 10, 15 or 25 nanomoles per liter depending on patient's response. The dosage needed to maintain these levels may vary from patient to patient. It is advisable for the attending clinical to monitor levels by the techniques described herein and optimize dosage accordingly. For prophylactic purposes, administration of the androgen is preferably started in the perimenopausal period for the prevention of breast and endometrial cancer and bone loss in normal women. The androgen may be associated with an accepted dose of an estrogen used to prevent other signs and symptoms of menopause. In women, when estrogen formation and/or action has been blocked for treatment of endometriosis, leiomyomata, breast cancer, uterine cancer, ovarian cancer or other estrogen-sensitive disease, administration of the androgen can be started at any time, preferably at the same time as blockade of estrogens.

The androgen for intramuscular or subcutaneous depot injection may be microencapsulated in a biocompatible, biodegradable polymer, e.g., poly(d,l-lactide-co-glycolide) by, among other techniques, a phase separation process or formed into a pellet or rod. The microspheres may then be suspended in a carrier to provide an injectable preparation or the depot may be injected in the form of a pellet or rod. See also European patent application EPA No. 58,481 published Aug. 25, 1982 for solid compositions for subdermal injection or implantation or liquid formulations for intramuscular or subcutaneous injections containing biocompatible, biodegradable polymers such as lactide-glycolide copolymer and active compounds. These formulations permit controlled release of the compound.

The androgens useful in the present invention can be typically formulated with conventional pharmaceutical excipients, e.g., spray dried lactose and magnesium stearate into tablets or capsules for oral administration.

The active substance can be worked into tablets or dragee cores by being mixed with solid, pulverulent carrier substances, such as sodium citrate, calcium carbonate or dicalcium phosphate, and binders such as polyvinyl pyrrolidone, gelatin or cellulose derivatives, possibly by adding also lubricants such as magnesium stearate, sodium lauryl sulfate, "Carbowax" or polyethylene glycol. Of course, taste-improving substances can be added in the case of oral administration forms.

As further forms, one can use plug capsules, e.g., of hard gelatin, as well as closed soft-gelatin capsules comprising a softener or plasticizer, e.g. glycerine. The plug capsules contain the active substance preferably in the form of granulate, e.g., in mixture with fillers, such as lactose, saccharose, mannitol, starches, such as potato starch or amylopectin, cellulose derivatives or highly dispersed silicic acids. In soft-gelatin capsules, the active substance is preferably dissolved or suspended in suitable liquids, such as vegetable oils or liquid polyethylene glycols.

In place of oral administration, the active compound may be administered parenterally. In such case, one can use a solution of the active substance, e.g., in sesame oil or olive oil. The active substance can be microencapsulated in or attached to a biocompatible, biodegradable polymer, e.g.

poly(d,l-lactide-co-glycolide) and subcutaneously or intramuscularly injected by a technique called subcutaneous or intramuscular depot to provide continuous slow release of the compound(s) for a period of 2 weeks or longer.

The invention also includes kits or single packages containing the pharmaceutical composition active ingredients or means for administering the same for use in the prevention and treatment of breast and endometrial cancer as well as bone loss and treatment of endometriosis as discussed above. The kits or packages may also contain instructions on how to use the pharmaceutical compositions in accordance with the present invention.

Following the above therapy using the described regimen, tumor growth of breast and endometrial cancer as well as bone loss and endometriosis can be relieved while minimizing adverse side effects. The use of the described regimen can also prevent appearance of the same diseases.

EXAMPLE 1

Prevention of Mammary Carcinoma Induced by Dimethylbenz(a)anthracene (DMBA) in the Rat, By Low Dose Medroxyprogesterone Acetate ("MPA")

To illustrate the efficacy of the present invention in reducing the incidence of mammary carcinoma, FIG. 1 illustrates the effect of a single subcutaneous injection of Depo-Provera (Medroxyprogesterone Acetate (MPA) (30 mg)) one week before inducing carcinoma with dimethylbenz(a)anthracene. FIG. 1 shows the period from 30 to 85 days following administration of DMBA. One curve in FIG. 1 shows the average number of tumors per animal in the group protected by Depo-Provera while the other curve shows the average number of tumors per animal in the unprotected group. It is estimated that the 30 mg. injection of Depo-Provera would release approximately 0.17 mg. of active medroxyprogesterone acetate per day over a six-month period. As may be seen by comparing the two graphs in FIG. 1, the Depo-Provera-treated group showed much greater resistance to the development of tumors than did the unprotected group. After 85 days an average of 1.89 tumors per rat was observed in the unprotected group, while only 0.30 tumor per rat was observed in the Depo-Provera protected group. Tumor number and size measured with calipers were determined weekly.

EXAMPLE 2

Treatment of Mammary Carcinoma Induced By Dimethylbenz(a)anthracene In the Rat, By Low Dose Medroxyprogesterone Acetate

FIG. 2 illustrates the inhibition of mammary carcinoma growth which may be achieved in accordance with the methods of the invention. Tumors were induced in ovariectomized rats using dimethylbenz(a)anthracene. Estradiol was used to stimulate growth in both a treatment and control group of rats. Each animal in the treatment group received a single subcutaneous administration of 30 mg of Depo-Provera (which is estimated to release approximately 0.17 mg. per day of active medroxyprogesterone acetate for a period of about six months). This figure illustrates the average estradiol-stimulated change in total tumor area in each group following treatment. As may be seen in FIG. 2, the group treated with Depo-Provera exhibited significantly less tumor growth than the untreated group.

The terms and descriptions used herein are preferred embodiments set forth by way of illustration only, and are

not intended as limitations on the many variations which those of skill in the art will recognize to be possible in practicing the present invention as defined by patent claims based thereon.

What is claimed is:

1. A method for treating osteoporosis in a patient in need of such treatment, comprising administering to said patient at least one androgenic steroid having a K_i value for the androgen receptor of less than about $2 \times 10^{-8} M$ and an androgen receptor-mediated inhibitory effect on the growth of human breast cancer ZR-75-1 cells which reaches half-maximal value at a concentration below 3.0 nanomoles per liter and no visible masculinizing activity wherein said androgenic steroid is administered at a dosage sufficiently low to maintain a cumulative androgenic steroid serum concentration between 1 and 50 nanomoles per liter for a period of at least 30 days.

2. The method of claim 1, wherein said cumulative serum concentration is maintained between about 1 and 25 nanomoles per liter.

3. The method of claim 1, wherein said cumulative serum concentration is maintained between 1 and about 15 nanomoles per liter.

4. The method of claim 1, wherein said cumulative serum concentration is maintained between 1 and 10 nanomoles per liter.

5. The method of claim 1, wherein said androgenic steroid(s) is(are) administered together with a pharmaceutically acceptable diluent or carrier in a dosage which delivers less than 25 milligrams of such androgenic steroid(s) per 50 kilograms of body weight per day.

6. The method of claim 5, wherein said androgenic steroid(s) is(are) administered in an amount between about 1 and about 10 milligrams of said androgenic steroid(s) per 50 kilograms of body weight per day.

7. The method of claim 5, wherein said androgenic steroid(s) is(are) administered in an amount between about 3 and about 7 milligrams of said androgenic steroid(s) per 50 kilograms of body weight per day.

8. The method of claim 1, wherein at least one androgenic steroid is a synthetic progestin.

9. The method of claim 1, wherein at least one androgenic steroid is an anabolic steroid.

10. The method of claim 1, wherein at least one androgenic steroid is medroxyprogesterone acetate (17 α -acetoxy-6 α -methyl progesterone).

11. The method of claim 7, wherein at least one androgenic steroid is medroxyprogesterone acetate (17 α -acetoxy-6 α -methyl progesterone).

12. The method of claim 11 further comprising administering an effective amount of estrogen for prevention or reduction of menopausal symptoms selected from the group

consisting of vasomotor symptoms, urogenital atrophy, bone loss, irritability, insomnia, anxiety and fatigue.

13. The method of claim 2 further comprising administering an effective amount of estrogen for prevention or reduction of menopausal symptoms selected from the group consisting of vasomotor symptoms, urogenital atrophy, bone loss, irritability, insomnia, anxiety and fatigue.

14. The method of claim 3 further comprising administering an effective amount of estrogen for prevention or reduction of menopausal symptoms selected from the group consisting of vasomotor symptoms, urogenital atrophy, bone loss, irritability, insomnia, anxiety and fatigue.

15. The method of claim 4 further comprising administering an effective amount of estrogen for prevention or reduction of menopausal symptoms selected from the group consisting of vasomotor symptoms, urogenital atrophy, bone loss, irritability, insomnia, anxiety and fatigue.

16. The method of claim 1 where osteoporosis is treated in a human patient.

17. The method of claim 16, wherein said cumulative serum concentration is maintained between about 1 and 25 nanomoles per liter.

18. The method of claim 16, wherein said cumulative serum concentration is maintained between 1 and about 15 nanomoles per liter.

19. The method of claim 16, wherein said cumulative serum concentration is maintained between 1 and 10 nanomoles per liter.

20. The method of claim 16 wherein said androgenic compound is medroxyprogesterone acetate.

21. The method of claim 16 wherein said androgenic compound is megestrol acetate.

22. The method of claim 16 wherein said androgenic compound is Gestodene.

23. A method for treating osteoporosis in a patient in need of such treatment comprising administering to said patient medroxyprogesterone acetate at a dosage sufficiently low to maintain serum concentration of said medroxyprogesterone acetate between 1 and 50 nanomoles per liter for at least 30 days.

24. The method of claim 23, wherein said dosage is between 1 and 10 milligrams per 50 kilograms of body weight per day.

25. A method for treating osteoporosis in a patient in need of such treatment comprising administering megestrol acetate to said patient at a dosage sufficiently low to maintain serum concentration of said megestrol acetate between 1 and 50 nanomoles per liter for at least 30 days.

26. The method of claim 25, wherein said dosage is between 1 and 10 milligrams per 50 kilograms of body weight per day.

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Testicular Heat Exposure Enhances the Suppression of Spermatogenesis by Testosterone in Rats: The "Two-Hit" Approach to Male Contraceptive Development*

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ABSTRACT

The objectives of the study were to determine stage-specific changes in the kinetics of germ cell apoptosis induced by administration of exogenous testosterone (T) alone and to examine whether addition of a single testicular heat exposure would enhance the induction of germ cell apoptosis and the suppression of spermatogenesis by T. Adult male rats were implanted with 3-cm SILASTIC brand capsules (Dow Corning Corp.) containing T for up to 6 weeks. Intratesticular T levels declined to 2.9% of control values by 1 week and remained suppressed at 2, 3, and 6 weeks after T administration. The incidence of germ cell apoptosis (expressed as numbers per 100 Sertoli cells) was low in control rats (0–9.52). After T treatment, the mean incidence of apoptosis at stages VII–VIII increased significantly by 1 week (21.43 ± 3.33) and showed further increases by 6 weeks (56.30 ± 7.47); apoptotic rates remained low at early (I–VI) and later (XII–XIV) stages. To test whether the combination of T with a single testicular heat exposure resulted in more complete suppression of spermatogenesis than either treatment alone, four groups of adult rats received one of the following treatments: 1) a subdermal empty polydimethylsiloxane implant, 2) exposure to a single testicular heating (43 C for 15 min) applied on day 14, 3) 3-cm T implant, or 4) 3-cm T implant and a single testicular heat exposure (applied on day 14). All animals were killed at the end of 6 weeks. In the heat-treated group, testis weight and testicular sperm counts were decreased to 65.4% and 28.9% of control

levels, respectively. The corresponding values in the T-treated group were 49.7% and 24.9% of control levels, respectively. Notably, addition of heat to T further reduced testis weight to 31.1% of control levels and testicular sperm counts to near zero. Histomorphometric analysis showed that all treatments reduced seminiferous tubular diameter and epithelial and luminal volume, with the greatest decrease after combined T and heat treatment. Heat exposure in animals bearing T implants markedly reduced the number of pachytene spermatocytes and round spermatids through apoptosis, resulting in tubules devoid of mature spermatids. Spermatogonia and preleptotene spermatocytes remained unaffected. These results clearly demonstrate that 1) exogenous T reduces intratesticular T and induces apoptosis mainly at stages VII–VIII within 1–6 weeks; 2) the combined treatment of T and heat markedly inhibits spermatogenesis, resulting in near azoospermia within 6 weeks; and 3) meiosis and spermiogenesis are the most vulnerable phases of spermatogenesis in response to T plus heat treatment. These findings suggest that a combination of hormonal treatment such as T and a physical agent (heat exposure) is more effective in suppressing spermatogenesis than either treatment alone. We hypothesize that combination of two antispermatogenic agents ("two hit") working at separate stages of the spermatogenic cycle will lead to greater male contraceptive efficacy. (*Endocrinology* 141: 1414–1424, 2000)

WE HAVE PREVIOUSLY demonstrated in the rat that stage-specific loss of germ cells occurred exclusively by apoptosis after acute withdrawal of gonadotropins and intratesticular T by GnRH antagonist treatment. The hormone-dependent stages VII–VIII were the first to show enhanced germ cell apoptosis, occurring 5–7 days after GnRH antagonist-induced suppression of gonadotropins and the resultant decrease in intratesticular T (1, 2). Preleptotene (PL) and pachytene (P) spermatocytes as well step 7 and step 19 spermatids were most susceptible to the lack of hormonal stimulation. Although presumed to be similar to that of GnRH antagonist (suppression of serum LH or FSH), the mode of cell death during spermatogenic suppression by

exogenous administration of testosterone (T) alone has not yet been characterized.

In additional studies (3), we further confirmed and extended earlier studies (3a) by demonstrating that a single exposure (43 C for 15 min) of the rat testis to heat resulted in selective, but reversible, damage to the seminiferous epithelium through increased germ cell apoptosis. Heat-induced germ cell apoptosis predominantly occurred at early (I–IV) and late (XII–XIV) stages. Spermatocytes, including P at stages I–IV and XII, diplotene and dividing spermatocytes at stages XIII–XIV, and early (steps 1–4) spermatids at stages I–IV, were most susceptible to heat. Stages V–VI and VII–VIII were relatively protected from heat-induced apoptosis. We have also provided evidence indicating that mild testicular hyperthermia is able to increase germ cell apoptosis at stages VII–VIII only when intratesticular T levels were decreased by the prior treatment with GnRH antagonist. We conclude from these studies that intratesticular T plays a pivotal role in protecting germ cells at stages VII–VIII against heat-induced cell death (3).

In clinical studies to develop male hormonal methods of contraception, T administration resulted in reversible sup-

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pression of spermatogenesis. Although quite effective, the suppression was not uniform, and azoospermia was achieved in 60–90% of men (4–6) only when serum T was elevated to the upper normal range. Moreover, the time required to achieve azoospermia or severe oligozoospermia usually takes more than 12 weeks. Although there are no data to support such a contention; concern has been expressed that high doses of T may have untoward effects on prostate. To develop new regimens that could rapidly induce development of azoospermia in all men with a lower dose of T, clinical studies were designed to combine T with progestins or GnRH analogs (7–9). Some of these combined regimens achieved azoospermia in over 90% of men in 8–12 weeks. Thus, even the combined T and progestogen regimens leave room for improvement to create a faster and more complete male contraceptive approach.

In our search for optimization of an experimental suppressor of spermatogenesis, we propose that a two-hit approach, with addition of heat treatment to exogenous hormone treatment such as T, will enhance the decrease in sperm output by inducing apoptosis at all stages of seminiferous epithelial cycle. When confirmed, the identification of the mechanisms by which hormonal and physical factors induce apoptosis at different stages involving different cell types will possibly allow the replacement of the specific heat-induced effects that target pharmacological agents. Such a combination would suppress sperm counts quickly and more completely.

The objectives of the present study were 1) to document the temporal and stage-specific changes in the kinetics of germ cell apoptosis induced by administration of exogenous T alone, and 2) to determine whether the combination of a single testicular heat exposure with administration of a low dose of T could enhance the effect of T alone in rapidly and effectively suppressing spermatogenesis to near-complete azoospermia.

Materials and Methods

Animals

Adult (60-day-old) male Sprague Dawley rats (280–350 g) purchased from Charles River Laboratories, Inc. (Wilmington, MA) were used in the study. Animals were housed in a standard animal facility under controlled temperature (22°C) and photoperiod (12 h of light, 12 h of darkness), with free access to water and rat chow.

T and heat treatment

T SILASTIC implants, 3 cm in length, were prepared from polydimethylsiloxane tubing (od, 3.18 mm; id, 1.98 mm; Dow Corning Corp., Midland, MI), packed with T (Sigma, St. Louis, MO), and sealed with SILASTIC medical adhesive A (Dow Corning Corp.) based on previously described methods (10, 11). The release rate of T from the same type of implant was estimated to be about 30 µg/cm·day (12). A 3-cm T-filled capsule was implanted subdermally along the dorsal surface of each rat under pentobarbital anesthesia and kept for different periods of time. Heating of the scrota of the adult rats was performed using a procedure described previously (3). Briefly, rats were anesthetized with an ip injection of sodium pentobarbital (40 mg/kg BW) and then placed in a specially constructed holder. The scrotum containing testes and the tail were then immersed for 15 min in a thermostatically controlled water bath at 43°C. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Harbor-University of California-Los

Angeles Research and Education Institute animal care and use review committee.

Study protocols

Exp 1 (T treatment alone). To document the temporal and stage-specific changes in the kinetics of germ cell apoptosis induced by exogenous administration of T, experimental animals (four or five in each group) were implanted subdermally with a 3-cm T capsule for 1, 2, 3, and 6 weeks, and control animals were implanted with a 3-cm empty capsule.

Exp 2 (T with or without concomitant heat application). In this experiment we examined whether the addition of a single testicular heat exposure to animals bearing T implant could result in more rapid and effective suppression of spermatogenesis than T alone. To establish the optimum time point of heat exposure, preliminary experiments were performed in which a single heat exposure was applied to the testis at 0, 1, 2, 3, and 4 weeks after insertion of the T implant. Animals were killed at end of 6 weeks. Based on preliminary data (see Results), subsequent experiments were performed where heat was applied 2 weeks after T implantation. Sixteen young adult male Sprague Dawley rats were randomly assigned to four groups to receive one of the following treatments. Group 1 (control) received a subdermal empty implant for 6 weeks. Group 2 (heat only) was exposed once to testicular heat of 43°C for 15 min, applied 2 weeks after empty capsule implantation. Group 3 (T only) was given a 3-cm T implant for 6 weeks. Group 4 (T+heat) received a subdermal T implant in combination with a single heat exposure applied 2 weeks later. All animals were killed at the end of 6 weeks.

Blood collection and tissue preparation

Both control and experimental animals were injected with heparin (130 IU/100 g BW, ip) 15 min before being killed by a lethal injection of sodium pentobarbital (100 mg/kg BW, ip) to facilitate testicular perfusion using a whole body perfusion technique (13, 14). Body weight was recorded at autopsy. Blood samples were collected from the inferior vena cava of each animal immediately after death, and plasma was separated and stored at –20°C for subsequent hormone assays. Also before perfusion, one testis from each rat was removed and weighed, and after decapsulation, testicular parenchyma were used for determining the number of advanced (step 17–19) spermatids by the homogenization technique (15). In brief, testicular parenchyma were weighed and then homogenized in the same volume (equivalent to testicular parenchyma weight) of 0.01 M PBS (pH 7.4). An aliquot, after appropriate dilution, was counted in a hemocytometer. Each square of the hemocytometer with coverslip in place represents a total volume of 10^{-4} cm³. Results were expressed as number of spermatids per ml or per g testis. The figure obtained was then multiplied by the testis volume (equivalent of testicular weight) to yield the number of spermatids per testis. The remaining homogenized aliquots of testicular parenchyma from each rat were kept frozen at –70 to –80°C until used for testicular T assay. The contralateral testes were then fixed by vascular perfusion with 5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 30 min, preceded by a brief saline wash. The ventral prostates and seminal vesicles were carefully dissected out and weighed. The testes were removed, cut into small (~0.2-cm) transverse slices, and placed into the same fixative overnight. One slice from the middle region of the testis was processed for routine paraffin embedding for *in situ* detection of apoptosis. The adjacent testicular slice from each rat was further diced into small pieces (1 × 2 × 2 mm), postfixed in 1% osmium tetroxide–1.25% potassium ferrocyanide mixture, dehydrated in a graded series of ethanol, and embedded in Araldite. Embedded testicular specimens were sectioned with an LKB ultramicrotome (Rockville, MD) at 2.05 µm and stained with 1% toluidine blue for light microscopic examination and morphometric studies (13).

Hormone assays

The T concentrations in plasma and testicular homogenates were measured by RIA, as reported previously (16). Testicular tissue was homogenized in PBS (pH 7.4). All samples were then extracted with 10 vol of a mixture of ethyl acetate-hexane (3:2, vol/vol) before RIA. The minimal detection limit in the assay was 0.25 ng/ml. The intra- and

interassay coefficients of variations were 8% and 11%, respectively. Plasma FSH levels were measured by RIA, using reagents provided by the NIDDK, as previously described (16). Rat (r) FSH RP-2 reference preparation and rFSH S-11 antiserum were used. The minimal detection limit in the assay was 0.4 ng/ml. The intra- and interassay coefficients of variations were 11% and 15%, respectively. Plasma LH levels were measured by an immunofluorometric assay for rLH (17) using a combination of monoclonal antibodies to human LH (Medix, Kauniainen, Finland) and bovine LH (provided by Dr. J. F. Roser, University of California-Davis), as described previously (16). The minimal detection limit in the assay was 0.02 ng/ml. The intra- and interassay coefficients of variation were 6% and 8%, respectively.

Assessment of apoptosis

In situ detection of cells with DNA strand breaks was performed in glutaraldehyde-fixed, paraffin-embedded testicular sections by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) technique using an Apop Tag-peroxidase kit (Oncor, Gaithersburg, MD). The choice of fixative was based on the results of our previous studies, which showed that glutaraldehyde fixation significantly improved both TUNEL specificity and sensitivity while maintaining excellent morphological preservation (2, 3, 18, 19). In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 15 min at room temperature, washed in distilled water, and then treated with 2% hydrogen peroxide in PBS for 5 min at room temperature to quench endogenous peroxidase activity. Sections were then incubated with a mixture containing digoxigenin-conjugated nucleotides and TdT in a humidified chamber at 37°C for 1 h and subsequently treated with antidigoxigenin-peroxidase for 30 min at room temperature. To detect immunoreactive cells, the sections were incubated with a mixture of 0.05% diaminobenzidine and 0.01% H_2O_2 for 6 min. Sections were counterstained with 0.5% methyl green, dehydrated in 100% butanol, cleared in xylene, and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

Negative and positive controls were carried out in every assay. As negative controls, tissue sections were processed in an identical manner, except that the TdT enzyme was replaced by the same volume of distilled water. Testicular sections from rats treated with GnRH antagonist for 7 or 14 days were used as positive controls (2).

Enumeration of the nonapoptotic Sertoli nuclei with distinct nucleoli and apoptotic germ cell population was carried out at stages I–IV, V–VI, VII–VIII, IX–XI, and XII–XIV using an Olympus Corp. BH-2 microscope with a $\times 100$ oil immersion objective. These stages were intentionally chosen not only to examine the whole seminiferous epithelial cycle, but also to focus attention on the heat- and hormone-sensitive stages (2, 3, 20–22). For each rat, at least 10 tubules/stage group were used. These stages were identified according to the criteria proposed by Russell *et al.* (23) for paraffin sections. The rate of germ cell apoptosis (apoptotic index) was expressed as the number of apoptotic germ cells per 100 Sertoli cells (2, 3).

Morphometric procedures

The volume densities (Vv) of seminiferous tubules, tubular lumens, interstitium, and Leydig cells were determined by a point-counting method (13, 14). Five randomly selected sections per animal in each group were examined by an American Optical Microscope (Scientific Instruments, Buffalo, NY) with a $\times 40$ objective and a $\times 10$ eye piece fitted with a square lattice containing 121 intersections. The results were expressed as a percentage of the testis volume. The absolute volume of each of the testis components was then obtained by multiplying its Vv by fresh testis volume (Vv%). The diameters of 20 randomly selected transverse sections of seminiferous tubules were measured across the minor axis of their profiles with an ocular micrometer calibrated by means of a stage micrometer.

Numerical densities (Nv) of Sertoli and germ cells (number per unit volume of the seminiferous tubule) at stages VII–VIII of the cycle was determined by accepted stereological techniques as described previously (13, 14). For each rat, 10 round cross-sections of seminiferous tubules were used. The Floderus equation $Nv = N_A / (T + D - 2h)$ was used to calculate the Nv of germ cell nuclei and Sertoli cell nucleoli, where N_A is the number of nuclei or nucleoli counted per unit area of

the seminiferous tubule profile, T is the section thickness, D is the average diameter of a given germ cell nucleus or the Sertoli cell nucleolus, and h is the height of the smallest recognizable nuclear or nucleolar profile in the section. The nuclear profile of each germ cell (A_1 spermatogonia, PL and P spermatocytes, and step 7 and 8 spermatids) and the number of Sertoli cell nucleoli (thereby cells, as only 1 typical nucleolus is present per nucleus) in the seminiferous tubules were counted under a $\times 1000$ magnification using an oil immersion objective. The seminiferous tubule profile area (a) was determined by point counting using the equation: $a = p \times u^2$, where p is number of points per tubular profile, and u is the distance between 2 neighboring point in terms of the magnification used to measure the area. The mean diameters of Sertoli cell nucleoli and germ cell nuclei were obtained by direct measurements of their largest cross-sectioned profiles in serial sections. Even though the profiles of A_1 spermatogonial nuclei were somewhat ellipsoidal, their eccentricity did not reach levels that would produce serious error. The height of the smallest recognizable nuclear or nucleolar profile was assumed to be 1/10th of the diameter of the structure. The Nv of a given cell type (number per unit volume of fixed tissue) was corrected by multiplying a factor of 0.855 to provide the number of cells per unit volume of the fresh tissue. The absolute number of these cells was then determined by multiplying their Nv by the fresh volume of the testis. Cell counts were finally expressed as the number of germ cells per Sertoli cell (germ cell/Sertoli cell ratios).

Statistical analysis

Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel Corp., San Rafael, CA). Results were tested for statistical significance using the Student-Newman-Keuls test after one-way repeated measures ANOVA. Differences were considered significant if $P < 0.05$.

Results

Exp 1: T alone

Body and organ weights and testicular sperm numbers. Body and organ weights and the number of homogenization-resistant advanced (step 17–19) spermatids in control and T-treated rats killed at various time intervals (1, 2, 3, and 6 weeks) are summarized in Table 1. No significant differences in the mean body weight and ventral prostate and seminal vesicle weights were observed among all treatment groups. In contrast, a significant ($P < 0.05$) decrease in testis weight was noted as early as 3 weeks after T administration. By 6 weeks, the mean testis weight was reduced to 57.8% of the values measured in controls. Mean testicular sperm content was also significantly decreased to 29.4% (10^6 /testis) of the control values at 6 weeks after T treatment.

Hormone levels. Hormone levels in controls and in the animals killed 1, 2, 3, and 6 weeks after T administration are summarized in Table 2. Plasma levels of FSH and LH decreased to 51.1% and 3.1% of control values, respectively, by 1 week and remained suppressed thereafter throughout the treatment period. Plasma T levels remained at the normal range throughout the entire treatment duration. Intratesticular T levels were rapidly and significantly reduced to 2.9% (ng/testis) of control levels as early as 1 week after T administration. No further decrease in intratesticular T levels was noted thereafter.

Stage-specific activation of germ cell apoptosis induced by T administration. To determine stage-specific changes in the kinetics of germ cell apoptosis induced by administration of exogenous T, we assessed the changes in the incidence of germ cell apoptosis at various time intervals after T treat-

TABLE 1. Body weight, organ weights, and testicular sperm numbers

	Control	Weeks after testosterone administration			
		1	2	3	6
No. of animals	4	5	5	5	4
BW (g)	319.6 ± 9.76	316.5 ± 5.58	347.9 ± 22.69	343.9 ± 16.05	425.2 ± 21.52
Ventral prostate wt (g)	0.57 ± 0.11	0.53 ± 0.11	0.58 ± 0.05	0.69 ± 0.06	0.79 ± 0.14
Seminal vesicle wt (g)	1.22 ± 0.21	1.17 ± 0.17	1.35 ± 0.24	1.38 ± 0.15	1.76 ± 0.22
Testis wt (g)	1.28 ± 0.03 ^a	1.33 ± 0.07 ^a	1.18 ± 0.05 ^a	1.03 ± 0.06 ^b	0.74 ± 0.03 ^c
Testicular sperm conc. (10 ⁶ /g)	92.5 ± 5.12 ^a	76.8 ± 4.41 ^a	70 ± 8.32 ^a	69.6 ± 5.49 ^a	47.6 ± 4.22 ^b
Testicular sperm content (10 ⁶ /testis)	118 ± 4.86 ^a	102.5 ± 7.13 ^{a,b}	83 ± 11.43 ^b	72 ± 8.06 ^b	34.7 ± 2.33 ^c

Values are the mean ± SEM. In each row, means with unlike superscripts are significantly different ($P < 0.05$).

TABLE 2. Effect of testosterone administration on plasma levels of FSH, LH, T, and testicular tissue levels of T in the adult rats

Hormone	Control	Weeks after testosterone administration			
		1	2	3	6
FSH (ng/ml)	7.78 ± 0.67 ^a	3.98 ± 0.24 ^b	4.05 ± 0.52 ^b	3.08 ± 0.28 ^b	2.88 ± 0.21 ^b
LH (ng/ml)	0.64 ± 0.08 ^a	0.02 ± 0.01 ^b	0.03 ± 0.02 ^b	0.02 ± 0.01 ^b	0.03 ± 0.01 ^b
PT (ng/ml)	2.25 ± 0.38	1.67 ± 0.16	1.57 ± 0.34	1.30 ± 0.06	1.55 ± 0.33
TT (ng/g)	398.6 ± 128.4 ^a	10.68 ± 1.08 ^b	12.70 ± 4.76 ^b	7.66 ± 0.15 ^b	5.28 ± 0.41 ^b
TT (ng/testis)	498.59 ± 149.46 ^a	14.50 ± 2.04 ^b	15.76 ± 6.76 ^b	7.87 ± 0.47 ^b	3.90 ± 0.43 ^b

Values are the mean ± SEM. PT, Plasma T; TT, testicular T. In each row, means with unlike superscripts are significantly different ($P < 0.05$).

TABLE 3. Apoptotic index (apoptotic germ cells per 100 Sertoli cells) at various seminiferous epithelial stages after testosterone administration

Stages	Control	Weeks after testosterone administration			
		1	2	3	6
I–IV	8.32 ± 1.87	5.69 ± 1.33	6.83 ± 0.49	4.91 ± 0.31	5.98 ± 2.11
V–VI	0	0	0	0	0
VII–VIII	0 ^a	21.43 ± 3.33 ^b	20.57 ± 2.19 ^b	37.29 ± 4.62 ^b	56.30 ± 7.47 ^c
IX–XI	1.59 ± 0.36 ^a	1.92 ± 0.22 ^a	2.29 ± 0.84 ^a	1.88 ± 0.49 ^a	5.99 ± 1.59 ^b
XII–XIV	9.52 ± 2.51	12.86 ± 2.47	7.94 ± 1.99	8.01 ± 0.84	6.55 ± 0.78

Values are the mean ± SEM. In each row, means with unlike superscripts are significantly different ($P < 0.05$).

ment. Changes in the incidence of germ cell apoptosis [apoptotic index (AI), expressed as number of apoptotic germ cells per 100 Sertoli cells] at various seminiferous epithelial stages are summarized in Table 3. A low incidence of germ cell apoptosis (AI = 1.59–9.52) was detected at stages I–IV, IX–XI, and XII–XIV in control rats. The incidence of germ cell apoptosis was significantly increased exclusively at stages VII–VIII at the earliest time point examined, 1 week (AI = 21.43) after T administration. A further increase in the incidence of apoptosis (AI = 56.30) was noted by 6 weeks, at which time the mean incidence of germ cell apoptosis became significantly elevated also at stages IX–XI (AI = 5.99). The earliest response (1 week) of the seminiferous epithelium to T implant involved primarily P spermatocytes and step 7 spermatids and occasionally step 19 spermatids at stages VII–VIII (Fig. 1, A–C).

Exp 2: combination of T treatment and heat application

In a preliminary set of experiments, changes in testis weight and the number of homogenization-resistant advanced spermatids were analyzed in T-treated rats that had been exposed to testicular heating applied at 0, 1, 2, 3, and 4 weeks after insertion of the T implant. Compared with individual treatment, T+heat (applied on week 2) clearly resulted in marked suppression of spermatogenesis, as ev-

idenced by testis weight and testicular sperm count (Table 4). Accordingly, this combined treatment regimen was used for subsequent experiments for detailed examination of apoptosis and histomorphometry. The data presented below represent the results of these experiments.

Testis weight, sperm number, and hormone levels. Testis weight (Fig. 2A) and testicular sperm numbers (Fig. 2B) were markedly decreased in both heat- and T-treated groups compared with those in control animals. Notably, T+heat further significantly reduced testis weight to 31.1% of control levels and specifically decreased sperm counts to almost zero. The effects of heat exposure and T administration, either alone or in combination, on plasma levels of FSH, LH, T, and testicular tissue levels of T in adult rats are summarized in Table 5. Plasma LH levels were decreased in the T and T+heat groups compared with those in either the control or heat-treated group. In contrast, plasma FSH levels were increased in the heat-treated group, but significantly decreased in both T and T+heat groups. Plasma LH and FSH levels were not different between T and T+heat groups. No significant difference in plasma T levels was noted among the groups. The total content of testicular T was markedly reduced in both T and T+heat groups compared with that in either the control

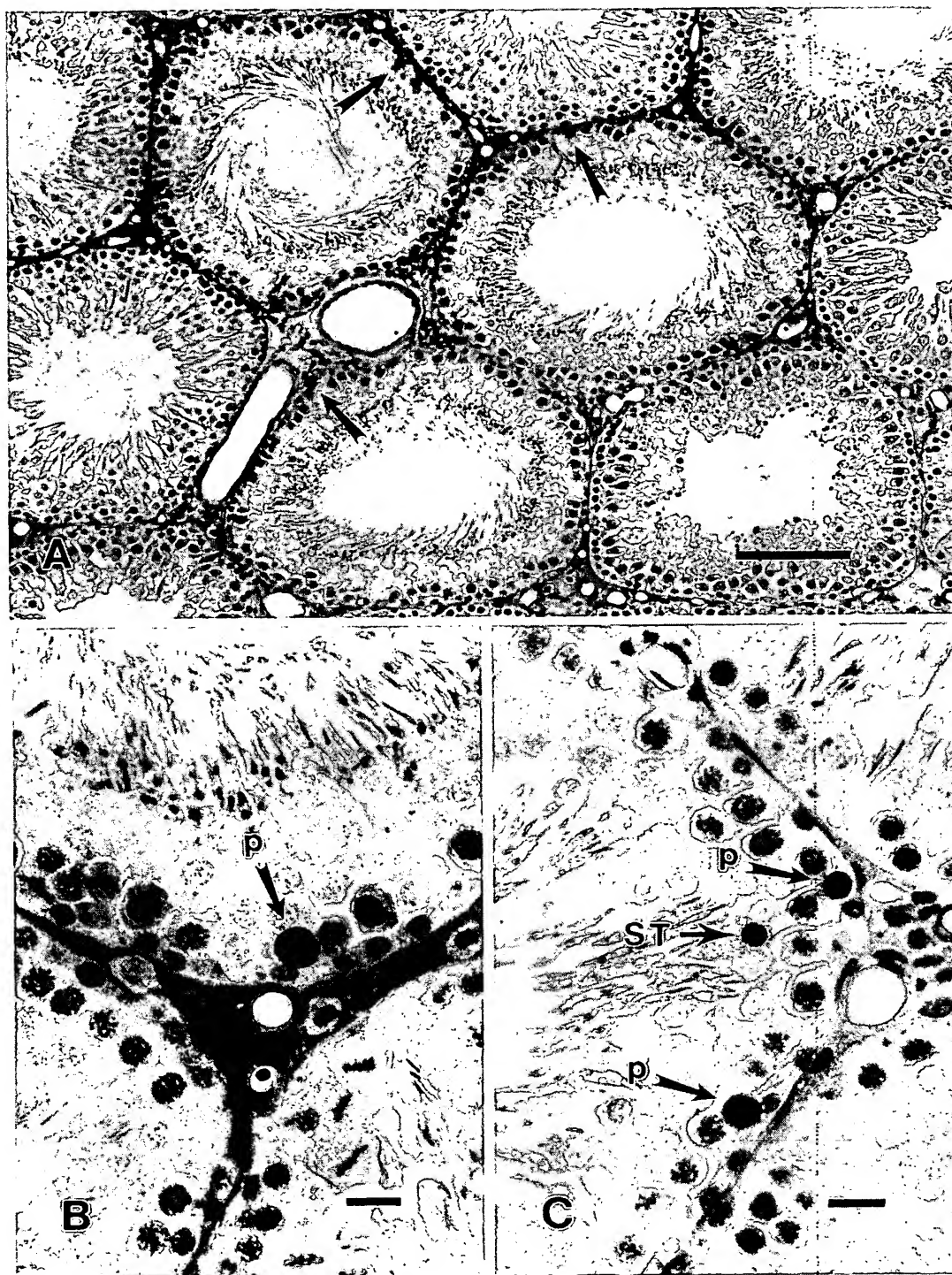


FIG. 1. *In situ* detection of germ cell apoptosis in rat testis. Cellular localization of apoptosis was characterized by TUNEL assay. Methylgreen was used as a counterstain. A, Low power light micrograph from a rat that received a 3-cm T implant for 1 week, showing increased germ cell apoptosis (arrow) at stage VII; such apoptotic germ cells are rarely if ever seen at stage VII in a control rat. B and C, Higher magnified views of portions of stage VII tubules from rats that received a 3-cm T implant for 1 week, exhibiting apoptotic P spermatocytes and a step 7 spermatid (ST). A: Magnification, $\times 180$; scale bar, 0.1 mm. B and C: Magnification, $\times 440$; scale bar, 0.02 mm.

TABLE 4. Effects of testosterone administration in combination with heat exposure applied at different time points on testis weight and testicular sperm numbers

	Control	Heat	T	Weeks applied heat exposure after insertion of T				
				0	1	2	3	4
No. of animals	4	4	4	4	4	4	4	4
Testis wt (g)	1.48 ± 0.07 ^a	1.01 ± 0.15 ^b	0.74 ± 0.06 ^c	0.55 ± 0.08 ^c	0.56 ± 0.26 ^c	0.46 ± 0.04 ^d	0.50 ± 0.05 ^c	0.48 ± 0.13 ^d
Testicular sperm conc. (10 ⁶ /g)	89.18 ± 6.12 ^a	60.9 ± 7.93 ^b	47.6 ± 4.22 ^b	2.46 ± 0.33 ^c	7.38 ± 3.01 ^c	0.21 ± 0.01 ^d	0.62 ± 0.39 ^d	5.28 ± 3.13 ^c
Testicular sperm content (10 ⁶ /testis)	131.92 ± 9.4 ^a	62.6 ± 12.4 ^b	34.7 ± 2.33 ^b	1.33 ± 0.14 ^c	5.24 ± 3.34 ^c	0.11 ± 0.01 ^d	0.33 ± 0.21 ^d	3.07 ± 1.81 ^c

Values are the mean ± SEM. In the heat alone group, heat exposure was applied on day 0. All the animals were killed at the end of 6 weeks. In each row, mean numbers with unlike superscripts are significantly different ($P < 0.05$).

Values are the mean ± SEM. In the heat alone group, heat exposure was applied on day 0. All the animals were killed at the end of 6 weeks. In each row, mean numbers with unlike superscripts are significantly different ($P < 0.05$).

or heat-treated group. No differences in intratesticular T levels were apparent between T and T+heat groups.

Testis morphology. Figure 3 shows the morphological appearance of the stage VII seminiferous tubules from a control (A), heat-treated (B), T-treated (C), and T- plus heat-treated (D) rats. In heat-treated animals (4 weeks after heat treatment), the majority of the seminiferous tubules revealed a picture of partial recovery of spermatogenesis (Fig. 3B). Histological examination further revealed a mild spermatogenic damage in T-treated animals, with the occurrence of degenerating germ cells in basal and midepithelial levels at stages VII–VIII (Fig. 3C). Compared with T or heat alone, T+heat clearly resulted in a marked impairment of spermatogenesis, and the seminiferous tubules were smaller in diameter and exhibited fewer P spermatocytes and round spermatids and complete absence of step 19 spermatids (Fig. 3D).

Morphometric observations. The results of the stereological analysis of the volumetric composition of the testes and tubule diameters among control, heat alone, T alone, and T in combination with heat exposure groups are summarized in Table 6. Treatment with either T or heat resulted in a significant decrease in seminiferous tubular diameter and the volume of the seminiferous tubules and their lumens, with the greatest decrease after T in combination with heat. The volumes of interstitium and Leydig cells were also markedly reduced both in T and T+heat groups compared with those in either the control or heat-treated group. No differences in volume of Leydig cells were apparent between T and T+heat groups.

Viable germ cell counts. The effects of T alone, heat alone, and T in addition to heat on spermatogenesis were further assessed by enumeration of the various germ cells (A1 spermatogonia, PL and P spermatocytes, and step 7 and 8 spermatids) supported per Sertoli cell at stages VII–VIII of the seminiferous epithelial cycle. No significant deviation in the number of Sertoli cells was noted among control ($19.10 \pm 2.18 \times 10^6$ /testis), heat-treated ($19.60 \pm 1.58 \times 10^6$ /testis), T-treated ($16.80 \pm 1.04 \times 10^6$ /testis), and T- plus heat-treated ($19.00 \pm 4.33 \times 10^6$ /testis) groups. No significant changes in the number of spermatogonia (Fig. 4A) and PL spermatocytes (Fig. 4B) were apparent among various treatment groups. There was, however, a significant decrease in the number of P spermatocytes (Fig. 4C) and round spermatids (Fig. 4D) in heat, T, and T+heat groups compared with controls. Notably, the number of P spermatocytes and round spermatids in T+heat group were decreased to 46.36% and 28.74% of control values, respectively, and also significantly decreased compared with those in either the heat or T treatment group.

In situ detection of germ cell apoptosis. As in the first experiment, exogenous administration of T for 6 weeks resulted in activation of germ cell apoptosis specifically at stages VII–VIII (Fig. 5A). In the heat alone group, the marked reduction of apoptosis was noted at early (I–IV) and late (XII–XIV) stages. The spermatogenesis had been partially recovered 4 weeks after heat exposure. In the T+heat group, the seminiferous tubules were smaller in diameter and exhibited a marked loss

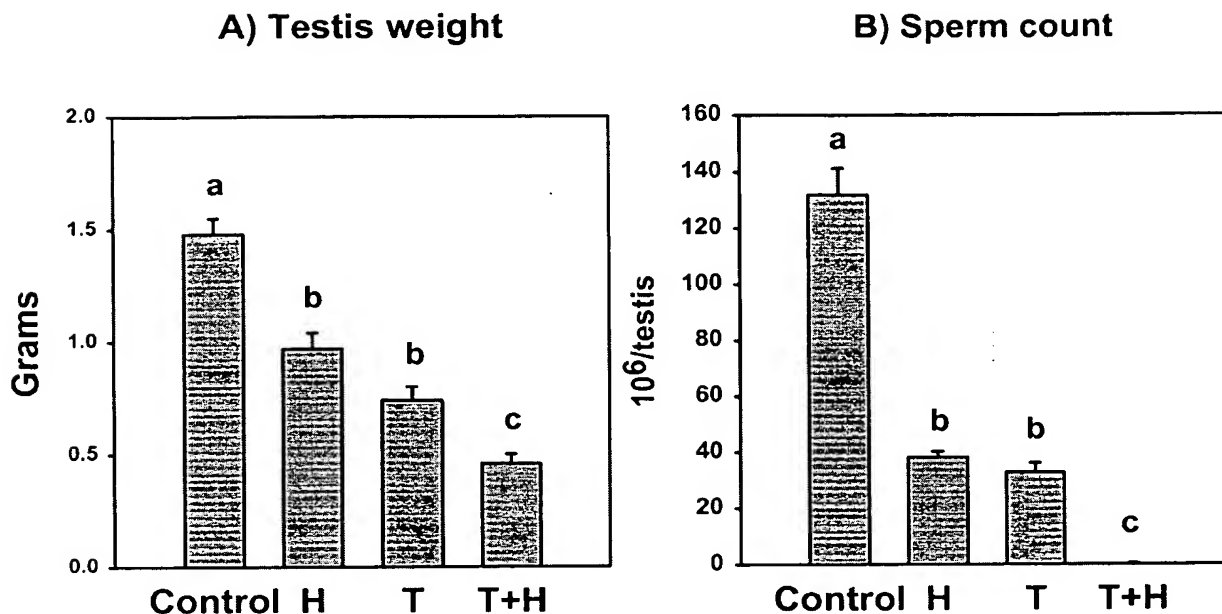


FIG. 2. Testis weight (A) and testicular sperm numbers (B) in control (C), heat only (H), T only (T), and T in combination with heat treatment (T+H) groups. The animals were killed 6 weeks after receiving T implants and 4 weeks after heat exposure. Heat or T alone significantly decreased testis weight and sperm count compared with control values. T in combination with heat was even more effective, resulting in a marked decrease in testis weight and reducing the sperm count to almost zero. Values are the mean \pm SD or SE. Means with unlike superscripts are significantly different ($P < 0.05$).

TABLE 5. Effects of heat alone, testosterone alone, and testosterone plus heat on plasma levels of FSH, LH, and T and testicular tissue levels of T in the adult rat

Hormone	Control	Heat	Testosterone	Testosterone + heat
FSH (ng/ml)	5.71 \pm 0.64 ^a	8.12 \pm 0.43 ^b	2.88 \pm 0.20 ^c	3.47 \pm 0.23 ^c
LH (ng/ml)	0.64 \pm 0.09 ^a	0.46 \pm 0.06 ^a	0.03 \pm 0.01 ^b	0.04 \pm 0.01 ^b
PT (ng/ml)	2.19 \pm 0.18	2.87 \pm 0.70	1.55 \pm 0.33	1.07 \pm 0.04
TT (ng/g)	416.05 \pm 52.24 ^a	466.68 \pm 118.34 ^a	5.28 \pm 0.41 ^b	11.29 \pm 1.47 ^b
TT (ng/testis)	611.83 \pm 64.67 ^a	463.40 \pm 127.56 ^a	3.90 \pm 0.43 ^b	5.20 \pm 0.68 ^b

Values are the mean \pm SEM. PT, Plasma T; TT, testicular T. In each row, means with unlike superscripts are significantly different ($P < 0.05$).

of germ cells. A few remaining apoptotic cells were detected in this group (Fig. 5B). Most of the apoptotic cells had been lost through phagocytosis by Sertoli cells. Spermatogonia and PL spermatocytes were unaffected.

Discussion

In this study, we demonstrated that the administration of a low dose of T to adult male rats results in the suppression of spermatogenesis, a decrease in intratesticular T, and preferential inhibition of plasma LH compared with plasma FSH. These events occur without significant changes in plasma T levels or weights of seminal vesicle and ventral prostate and confirm previous data from our laboratory (24, 25) as well as from others (11, 26–28). Intratesticular T was markedly decreased as early as 1 week and remained suppressed 2, 3, and 6 weeks after T implantation (11). We also demonstrated that administration of low dose of T induces activation of germ cell apoptosis involving mainly P spermatocytes and round spermatids at stages VII–VIII as early as 1 week. At 6 weeks, the incidence of germ cell apoptosis at stages VII–VIII is increased 2.6-fold over the 1 week treatment value. However,

by this time, stages IX–XI also exhibited a significant increase in the number of apoptotic germ cells. This is similar to our previous study in GnRH-antagonist treated rats (1, 2), in which stages VII–VIII were affected first, followed later by stages IX–XI. The occurrence of apoptotic germ cells at later stages might be a delayed consequence of abnormal development of germ cells as they pass through stages VII–VIII (2, 13, 29) or the consequence of an additive effect of FSH withdrawal. There is ample evidence to suggest that stages VII–VIII of the rat spermatogenic cycle exhibit the highest levels of immunocytochemically detectable androgen receptor expression and are considered to be androgen-dependent stages (29, 30).

We previously demonstrated that a single transient local testicular heat exposure induces germ cell apoptosis in a stage-specific and cell-specific fashion. Early (I–IV) and late (XII–XIV) stages are more sensitive to heat. We also demonstrated in that study that a combination of a single heat exposure with selective deprivation of gonadotropins and intratesticular T by GnRH antagonist treatment further results in the activation of apoptosis at both hormone- and

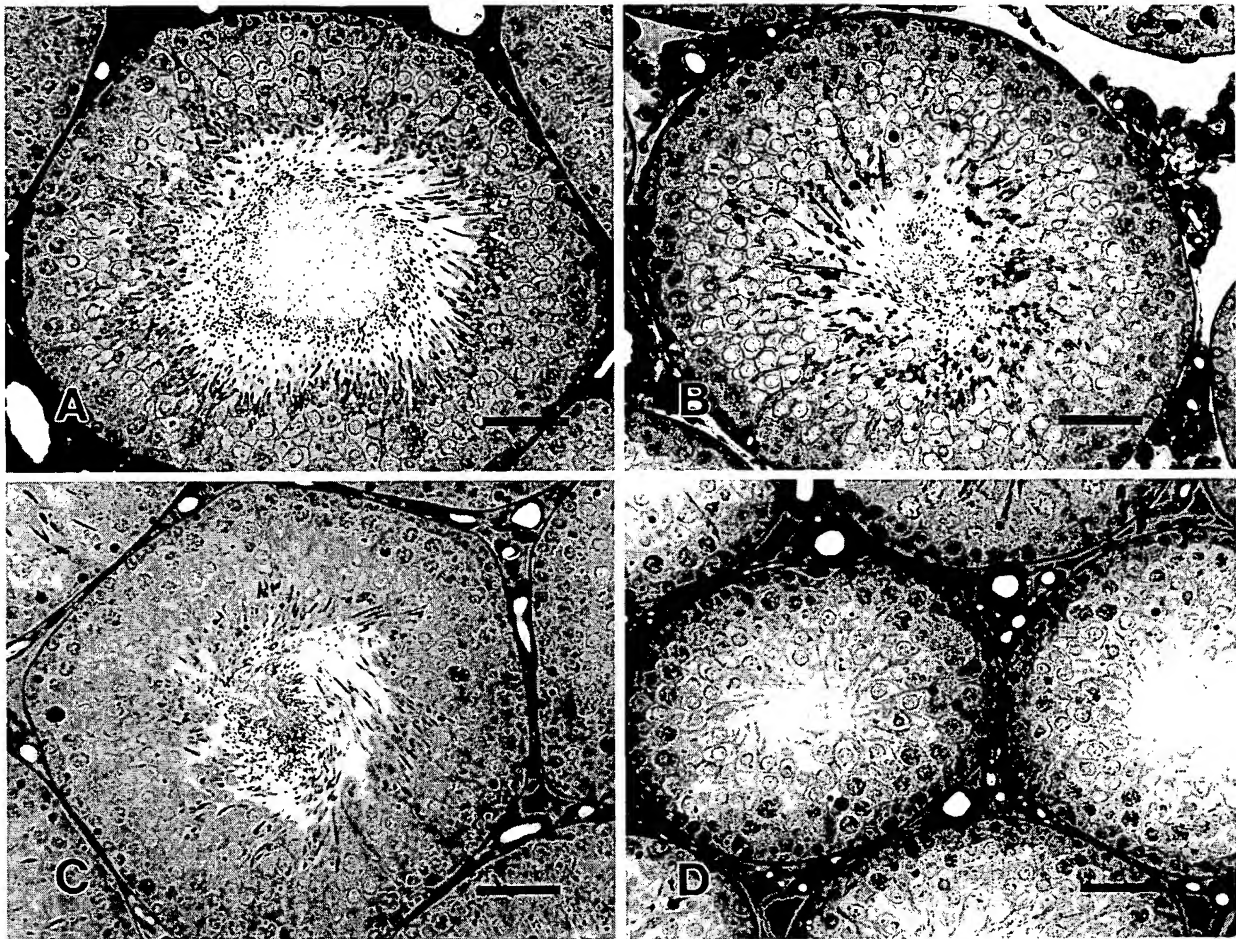


FIG. 3. Representative light micrographs of stage VII tubules from control (A), heat-treated (B), T-treated (C), and T- plus heat-treated (D) rats. Testes were fixed by vascular perfusion with 5% glutaraldehyde, postfixed in a 1% osmium-1.25% potassium ferro-cyanide mixture, and embedded in Araldite. T in combination with a single testicular heat exposure led to severe impairment of spermatogenesis (D). Note the marked reduction in the number of pachytene spermatocytes and step 7 spermatids and the complete absence of step 19 spermatids in the T+ heat group. Magnification, $\times 250$; scale bar, 0.05 mm.

heat-sensitive stages (3). Thus, we hypothesized that the combination of these two proapoptotic signals (hormone deprivation and heat stress) may surpass the efficacy of each individual signal and result in a rapid and marked suppression of spermatogenesis to complete azoospermia. Results obtained from the second experiment supported this hypothesis. Compared with individual apoptotic stimuli given alone (either heat or T treatment), T in combination with heat clearly resulted in marked suppression of spermatogenesis to almost azoospermia. The observed azoospermia most likely is attributed to increased germ cell apoptosis and their eventual phagocytosis by Sertoli cells. The reasons why T in combination with heat results in marked suppression of spermatogenesis can be best explained as follows. Exogenous T (hit 1) suppresses LH and FSH levels, lowers intratesticular T levels, and allows apoptosis at a moderate rate to occur in the hormone-sensitive (VII–VIII) stages without affecting heat-sensitive stages (I–IV and XII–XIV). The addition of heat

exposure (hit 2) to the testes with low intratesticular T levels induced by exogenous T administration results in marked acceleration of apoptosis at both hormone- and heat-sensitive stages, *i.e.* the window of protection seen with heat treatment alone had disappeared with prior hormone administration. Heat exposure accelerated the apoptosis caused by T treatment alone. In addition, we provided evidence indicating that the increased programmed germ cell death was independent of suppression of spermatogonia proliferation, as shown in this study and also demonstrated earlier in GnRH antagonist-treated or GnRH-immunized rats (13, 31, 32). This suggests that spermatogenesis inhibited by T in combination with heat will most likely attain full recovery after withdrawal of treatment. Unlike previous reports of combined treatment of T and estradiol in rats (28, 33), we did not observe sloughing of germ cells in the present study with T implants. This observation is consistent with our previous finding that no sloughing of germ cells occurred even after

TABLE 6. Effects of heat alone, testosterone alone, and testosterone plus heat on tubular diameter and on the volumetric composition of testis in rats

Parameters	Control	Heat	Testosterone	Testosterone + Heat
Tubule diameter (μm)	285.5 \pm 7.17 ^a	238.2 \pm 4.04 ^b	217.9 \pm 2.26 ^c	168.8 \pm 1.42 ^d
Vv% of testicular components				
ST	89.1 \pm 1.09 ^a	79.9 \pm 1.33 ^b	91.4 \pm 0.23 ^a	82.9 \pm 2.44 ^b
LU	13.1 \pm 0.49 ^a	7.58 \pm 0.10 ^b	10.7 \pm 0.24 ^b	7.77 \pm 1.10 ^b
IT	10.92 \pm 1.08 ^a	20.08 \pm 1.33 ^b	8.58 \pm 0.23 ^a	16.88 \pm 2.22 ^b
LC	3.57 \pm 0.29 ^a	3.79 \pm 0.29 ^a	1.46 \pm 0.12 ^b	2.09 \pm 0.16 ^b
Vol/testis				
ST (ml)	1.31 \pm 0.03 ^a	0.77 \pm 0.03 ^b	0.67 \pm 0.03 ^c	0.38 \pm 0.01 ^d
LU (μl)	194.2 \pm 10.68 ^a	73.2 \pm 2.54 ^b	79.0 \pm 4.09 ^b	36.2 \pm 4.44 ^c
IT (μl)	162.0 \pm 17.46 ^a	199.0 \pm 15.45 ^a	61.9 \pm 0.71 ^b	78.4 \pm 13.33 ^b
LC (μl)	52.95 \pm 5.12 ^a	36.79 \pm 3.72 ^b	10.73 \pm 0.91 ^c	9.55 \pm 0.46 ^c

Values are the mean \pm SEM. ST, Seminiferous tubule; LU, tubular lumen; IT, interstitium; LC, Leydig cells. Vv%, volume density (which is the volume of a given testicular component per unit volume of the testis) expressed as a percentage of the testis. In each row, mean numbers with unlike superscripts are significantly different ($P < 0.05$).

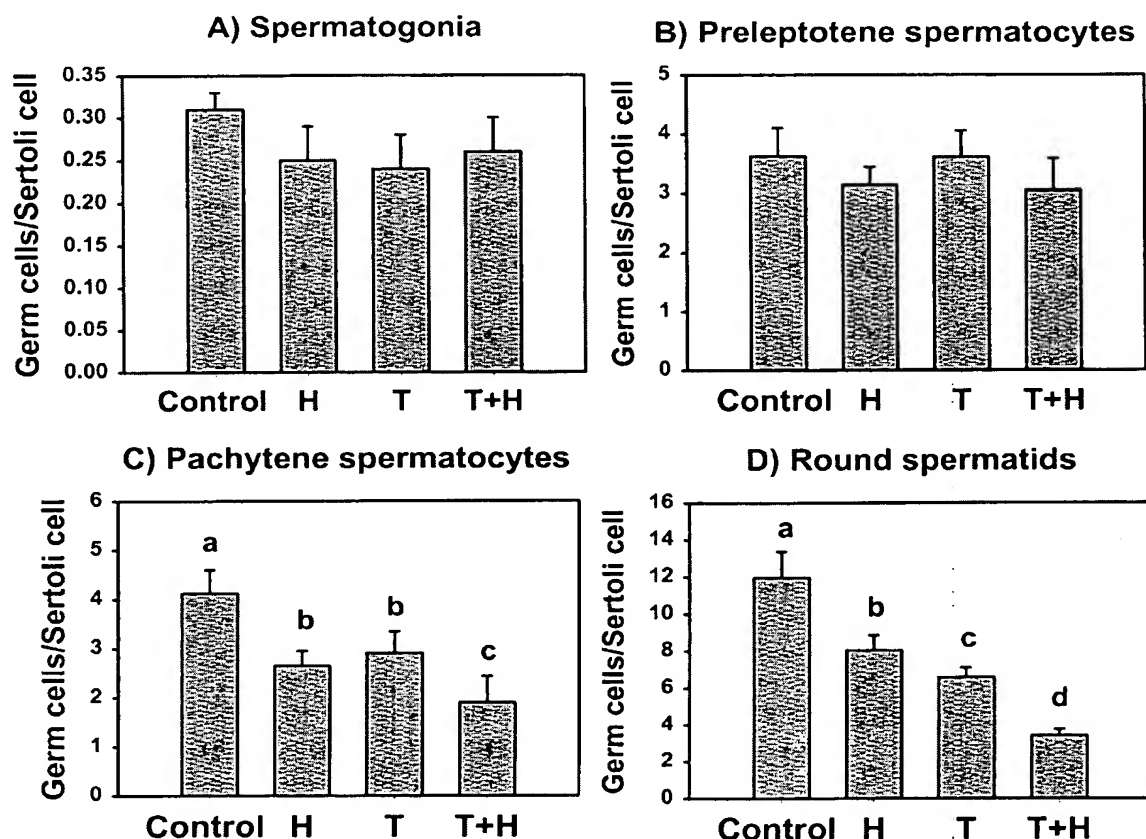


FIG. 4. Effects of T, either alone or in combination with heat, on germ cell-Sertoli cell ratios in rats. C, Control; H, heat only; T, T only; T+H, T in combination with heat. No changes in numbers of spermatogonia (A) or preleptotene spermatocytes (B) were observed among the groups. Either heat or T alone could significantly reduce the numbers of pachytene spermatocytes (C) and round spermatids (D). Note the marked reduction in the number of pachytene spermatocytes and round spermatids at stages VII–VIII in the T+heat group compared with those in the other groups.

4 weeks of GnRH antagonist treatment when the rat became completely azoospermia (2, 13). As expected (11), treatment with a low dose of T alone does not adequately withdraw the hormonal support, FSH in particular, required for optimum

suppression of spermatogenesis. At present we are unable to determine the precise mechanism that causes the much greater proportional decrease in the level of LH compared with FSH after exogenous T administration. However, it is



FIG. 5. Representative example of TUNEL of apoptotic germ cells in T alone (A) or T+heat (B) groups. A, Stage VII tubules from a T-treated rat for 6 weeks showing multiple apoptotic germ cells. B, Tubular profiles from a rat that received the combined treatment of T plus heat, showing marked regression of spermatogenesis. Note the marked decrease in tubule diameter and in the overall number of germ cells. A few remaining apoptotic germ cells can also be seen in these regressed tubules. Magnification, $\times 250$; scale bar, 0.05 mm.

clear that the marked suppression of spermatogenesis in the heat- plus T-treated group is most likely not influenced by gonadotropins and intratesticular T, as these parameters are not different between the T and T+heat groups.

The mechanisms by which these hormonal and nonhormonal factors govern germ cell apoptosis are not well understood. It is likely that apoptosis will be regulated in a cell type-specific fashion, but the basic element of the death machinery may be universal. A distinct genetic pathway is apparently shared by all multicellular organisms (34). The Bcl-2 family of proteins, which contains both proapoptotic (such as Bax) and antiapoptotic (such as Bcl-2) family members, constitutes a central checkpoint within this pathway. Bcl-2 and Bax have also been implicated as potential modulators of germ cell apoptosis (35). It has been reported that some members of the Bcl-2 family are involved in apoptosis after withdrawal of androgen support of the testis after treatment with ethane dimethanesulfonate, a Leydig cell cytotoxin (36). The Fas system is also a widely recognized apoptosis signal transduction pathway in which a ligand-receptor interaction triggers the cell death pathway. This system has recently been implicated in the activation of germ cell apoptosis in response to a variety of proapoptotic stimuli, including testicular hyperthermia and T withdrawal (37, 38). The present study did not address whether the same molecular mechanisms by which testicular hyperthermia or androgen withdrawal induces germ cell apoptosis are involved. Ongoing additional studies will elucidate the roles of Bax, Bcl-2, Fas, Fas ligand, and caspases in germ cell apoptosis triggered by these hormonal and nonhormonal regulatory stimuli.

These studies of the induction of germ cell apoptosis in rats are very likely applicable to humans. We and others have also demonstrated that spontaneous loss of germ cells occurs by apoptosis in adult human testis (39, 40). Other data suggesting a role for intratesticular T in suppressing apoptosis

in the human testes include the following: cessation of hCG treatment for cryptorchidism in prepubertal life increases apoptosis in the human testis (41); T regulates apoptosis in adult human seminiferous tubules *in vitro* (42); and apoptotic germ cells are present in testes from patients with prostate cancer who received short term antiandrogen treatment (43). Heat has long been recognized as a risk factor responsible for decreased sperm counts in men. Efforts have been made to harness these effects as an antifertility measure. Unfortunately, the effects have been incomplete or transient. Our group showed that an increase in human scrotal temperature of 0.8–1 C induced by polyester-lined athletic supports is insufficient to cause significant suppression of spermatogenesis or alteration of sperm function (44). We believe that this is due to failure to attain the critical (43 C) testicular temperature (45, 46) and the maintenance of normal or near-normal levels of intratesticular T after heat exposure. Based on the data provided from this study, in which administration of a low dose of T (hit 1) in combination with testicular warming (hit 2) rapidly suppresses spermatogenesis in rats, we postulate that the combination of hormone deprivation and heat could be applied as an induction strategy in the human. The advantages of this combination will be 1) shortening the duration of onset of suppression of spermatogenesis, resulting in azoospermia; and 2) lowering the dosage of T and diminishing the potential adverse effects of higher doses of T administration on prostate. In addition, the concept of a two-hit strategy will undoubtedly lead to fundamental studies of the mechanisms responsible for the induction of germ cell apoptosis by distinct pathways. This, in turn, will allow targeting of these pathways by pharmacological means to result in more rapid and complete azoospermia. Thus, this concept of the additive or synergistic effects of two distinct stimuli inducing germ cell apoptosis probably by different molecular cascades may have important applications for male contraceptive development.

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Original Paper

Localization of androgen receptor expression in human bone marrow

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Abstract

Androgens have been shown to modulate the haematopoietic and immune systems and have been used clinically for stimulating haematopoiesis in bone marrow failure conditions. To identify the bone marrow cell types as potential targets of androgens, an androgen receptor (AR)-specific antibody was used to localize the AR in normal human bone marrow biopsies. The results show that AR was ubiquitously expressed in the bone marrow of both males and females. Furthermore, the AR expression pattern did not change with age. Stromal cells, macrophages, endothelial cells, myeloblasts, myelocytes, neutrophils, and megakaryocytes expressed AR. In contrast, AR was not detected in the lymphoid and erythroid cells, or in eosinophils. These results indicate that androgens may exert direct modulating effects on a wide spectrum of bone marrow cell types via AR-mediated responses. Copyright © 2000 John Wiley & Sons, Ltd.

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Introduction

The androgen receptor (AR) is a member of the nuclear receptor superfamily [1]. It acts as a transcription factor for a variety of genes by binding to an androgen response element (ARE). The AR is activated by testosterone or 5 α -dihydrotestosterone (5 α -DHT) [2]. A variety of factors, including androgens, have been implicated in modulating AR expression during fetal development, sexual maturation, ageing, and malignant transformation. In many tissues, AR expression is down-regulated by androgens. In contrast, androgens up-regulate AR expression in other cell types, such as osteoblasts [3].

Before the recombinant haematopoietic growth factors became available, androgens were the main pharmacological agents for stimulating erythropoiesis. Their erythropoietic activity primarily involves the

stimulation of renal production of erythropoietin (Epo) [4]. Furthermore, clinical observations indicate that other effects also occur. For example, in some aplastic anaemia patients treated with androgens, resolution of anaemia is accompanied by granulocyte and platelet count improvement [5]. Androgens also have direct stimulatory effects on the pluripotent stem cell, as demonstrated by the improved recovery of haematopoiesis in irradiated mice given testosterone [6].

AR expression has been demonstrated in many tissues. Male sexual organs show strongly positive staining for AR, whereas other tissues, including hepatic, thyroid, pancreatic, gastrointestinal, renal, neuronal, muscular, and female reproductive organs, have weak, albeit positive, nuclear staining [2,7,8]. The only tissue that does not stain for AR is the spleen [2]. The expression of AR in bone and bone marrow endothelial [9] and mononuclear cells [10,11] has been

demonstrated. AR is also present at low densities in osteoblasts [12] and in the nuclei and the perinuclear area of mouse osteoclast-like cells [13]. In addition, AR has been found in immature murine B cell lines, marrow stromal cell lines [11], and human macrophage-like synoviocytes [14]. More recently, human megakaryocytes and platelets have been shown to contain AR, which was regulated by testosterone [15]. Finally, ligand-binding assays have shown the presence of a receptor in erythroblasts that has the physiochemical characteristics usually attributed to AR [4,16].

To support the evidence for direct action of androgens on bone marrow, we used a polyclonal antibody preparation specific to the human AR to localize AR in human bone marrow *in situ*. Furthermore, although normal ageing in the male is accompanied by a decline in the serum levels of testosterone [17], little is known as to how this decline might affect androgen-target organs. To determine whether AR expression decreases with age, we also examined different age groups.

Materials and methods

Human tissues

Preserved bone marrow biopsies of individuals with normal bone marrow pathological review were obtained from the surgical pathology files at the University of Rochester Medical Center. A total of 25 cases were examined (9 females and 16 males). The ages of the individuals ranged from 18 months to 92 years. Sections of prostate, lymph node, tonsil, and bone marrow were used as positive controls and bone marrow as negative controls.

Antibodies and reagents

Primary antibodies

Immunolocalization of AR was performed using a specific rabbit polyclonal antibody (NH27). This antibody has been characterized for its specificity [18–20]. It does not cross-react with oestrogen, progesterone, or glucocorticoid receptors. Endothelial cells were immunolocalized using a rabbit polyclonal antibody against human Factor VIII-related antigen (DPC, Los Angeles, CA, USA) and a mouse monoclonal antibody (MAb) against human CD31 (DAKO, Carpinteria, CA, USA). The latter antibody was also used for the immunolocalization of megakaryocytes. Macrophages were identified using a mouse MAb against human CD68 (DAKO). A mouse MAb against human glycophorin A (GP-A; DAKO) was used for identifying erythroid cells. Myeloid cells were identified by a mouse MAb against human CD33 (DAKO). Finally, stromal cells were immunolocalized by a mouse anti-human vimentin MAb (a generous gift of DPC).

Secondary antibodies

A biotinylated goat anti-rabbit IgG antibody (DPC or Vector Laboratories, Burlingame, CA, USA) was used

as the secondary antibody for the polyclonal NH27 antibody and the polyclonal antibody against human Factor VIII-related antigen. A biotinylated horse anti-mouse IgG antibody (Vector Laboratories) was used as the secondary antibody for the anti-CD31, anti-CD68, anti-CD33, and anti-GP-A MABs, and a biotinylated rabbit anti-mouse IgG antibody (DPC) was used for the anti-vimentin MAB.

Reagents

Horse radish peroxidase (HRP)-labelled streptavidin (DPC or Jackson, Westgrove, PA, USA) was used to detect the biotinylated secondary antibodies.

Immunohistochemistry

Bone marrow biopsies were decalcified, formalin-fixed, paraffin-embedded, and serially cut at 4–5 µm thickness. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 6 min, followed by a phosphate buffer (pH 7.4) rinse. Depending on the primary antibody type, the thin sections (a) were pepsin-treated (anti-CD68 and anti-human Factor VIII-related antigen) for 15 min with 0.16 g/l pepsin (Sigma, St. Louis, MO, USA) in 0.1 N HCl; or (b) underwent antigen unmasking with heat retrieval (NH27, anti-CD31, anti-CD33, anti-GP-A, and anti-vimentin) for 30 min immersed in a citrate buffer (pH 6.0) in a microwave pressure cooker at a high power setting. Non-specific binding was prevented by incubation with a suppressor (normal horse or normal rabbit sera for monoclonal primary antibodies and normal goat serum for polyclonal primary antibodies) for 15 min. The appropriately diluted primary antibody (1 : 500 for NH27, 1 : 25 for anti-CD31, 1 : 100 for anti-CD68, 1 : 25 for anti-CD33, 1 : 25 for anti-GP-A, and prediluted anti-vimentin and anti-human Factor VIII-related antigen) was applied and the slides were incubated at room temperature for 1 h (the NH27 antibody was incubated at 37°C) in a humid chamber, followed by incubation with the appropriate secondary antibody (titrated according to the manufacturer's instructions) for 30 min at room temperature. Finally, the slides were incubated with streptavidin-HRP (titrated according to the manufacturer's instructions) for 30 min at room temperature in the humid chamber. Peroxidase activity was localized with a 5 min incubation with 3-amino-9-ethylcarbazole (AEC; ScyTek, UT, USA), yielding red-brown staining. The slides were counterstained in Mayer's haematoxylin and eosin (H&E) and blued with 0.3% ammonia water. Photographs were taken using a microscope (Zeiss, Thornwood, NY, USA).

Negative controls were carried out by following the same protocol with the exclusion of the primary antibody incubation step.

Histological staining

Paraffin thin sections of the bone marrow biopsies were stained with Mayer's H&E (Sigma) for characterizing cell morphology.

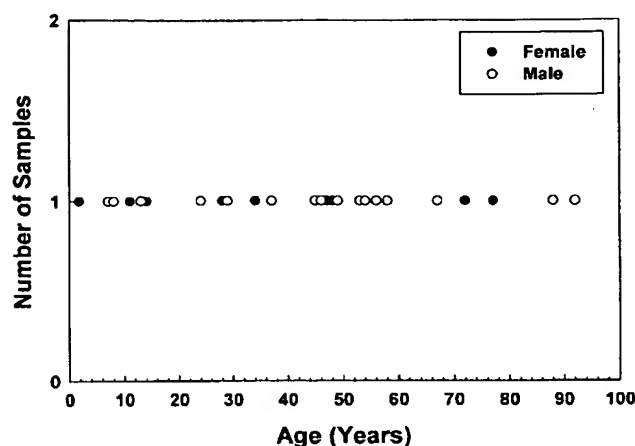


Figure 1. Sex and age distribution of the bone marrow biopsies used in this work (age 1–92 years; $n=25$, 16 males and 9 females)

Evaluation of specimens

Bone marrow biopsy slides were evaluated by three individuals. A consensus was obtained on morphological features, cell types, and AR staining.

Results

Bone marrow biopsies of 25 individuals with reported normal bone marrow on regular pathological review were examined. Sixteen of the biopsies were from males (7–92 years old) and nine from females (18 months to 77 years old), as shown in Figure 1.

The AR expression in human bone marrow was ubiquitous and extensive (Figure 2a). Both stromal and haematopoietic cells stained positively for AR, confirming previous reports [9]. Furthermore, AR was detected in the nucleus and the cytoplasm. There were no noticeable differences in the expression of AR between males and females. In addition, the AR expression pattern and uniformity did not appear to change with age, although cellularity did decrease in older individuals, as expected.

Focusing on bone marrow stroma, we confirmed AR expression in the stromal cells (Figure 2b), macrophages (Table 1), and endothelial cells (Figure 2c). AR expression in these cell types was localized mainly to the nucleus. In the haematopoietic compartment, AR was predominantly and highly expressed in neutrophils (Figure 2d). Both band and segmented neutrophils expressed high levels of nuclear and cytoplasmic AR. In contrast, nucleated erythroid cells (Figure 2d) and mature, enucleated red blood cells (Figures 2f and 2g) did not express AR.

Myelocytes and myeloblasts also expressed AR. In myelocytes, AR was mainly localized in the cytoplasm and showed perinuclear dot staining, morphologically similar to the expected location of the Golgi apparatus (Figure 2e). Similarly, myeloblasts expressed AR in

their cytoplasm (Table 1), but without the perinuclear dot staining. Weak perinuclear dot AR staining was observed in megakaryocytes (Figure 2f), but AR was not localized in the nucleus. Eosinophils did not express AR (Figure 2g). The presence of AR in basophils could not be determined (Table 1). Finally, lymphoid cells, both lymphoblasts and mature lymphocytes, did not appear to express AR. Figure 2h shows a benign lymphoid nodule that lacks AR expression. Table 1 summarizes the results of AR expression for the different cell types. In the negative controls, in which the primary antibodies were omitted, no staining was observed, whereas in positive controls (using the prostate, tonsil, lymph node, and bone marrow sections), the immunoreactivity of the antibodies used was confirmed.

Discussion

Our results demonstrate that AR expression is ubiquitous and extensive in the bone marrow of both males and females of all ages. This provides further evidence for the direct action of androgens on bone marrow and offers clues to clinicopathological correlates.

Testosterone levels in males are higher than in females (approximately ten times) [5]. Interestingly, we observed no noticeable differences in AR expression between males and females, suggesting that AR is able to mediate the direct effects of androgens on haematopoiesis in both sexes. Furthermore, although normal ageing in males is accompanied by a decline in the serum levels of testosterone, AR expression in bone marrow did not appear to decrease with age, in contrast to an age-dependent decline in the AR expression in rat liver [21]. This observation has potentially significant clinical implications, not only for the therapy of cytopenic conditions, but also for the mechanism of marrow failure states in all ages.

Androgens have been shown to regulate skeletal metabolism. In men and experimental animals, orchidectomy results in a rapid increase in the rate of bone remodelling and progressive bone loss. Osteoblasts have been reported to express the AR and exposure to androgens increases the steady-state AR mRNA levels [3]. AR expression in osteoclasts has been controversial. The presence of AR in mouse osteoclast-like cells *in vitro* has been reported [13], whereas AR expression in human osteoclasts has not been observed [9]. The androgen regulation of osteoclastogenesis and bone mass may be partially due to the indirect action of androgen via interleukin-6 (IL-6) [22]. Both stromal and osteoblastic cells produce IL-6. The action of androgens on osteoclasts may, therefore, be mediated indirectly through the interactions between the stromal cells and osteoclasts in bone marrow. The expression of AR in bone marrow stromal cells and endothelial cells was demonstrated in our work and is consistent with observations from others [9].

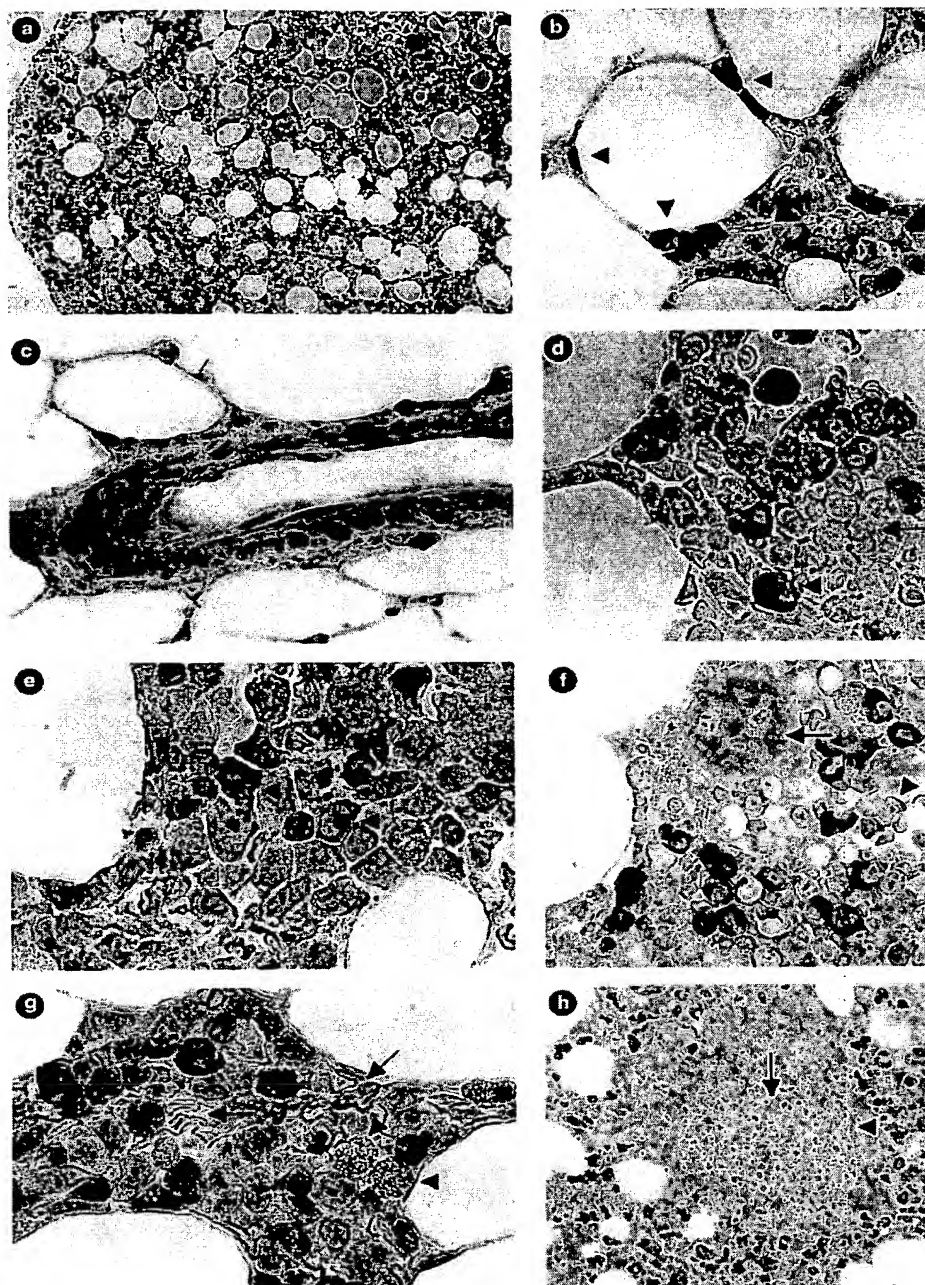


Figure 2. Localization of the AR in formalin-fixed, paraffin-embedded thin sections from bone marrow biopsies of normal specimens. Panel a shows the ubiquitous expression of AR in bone marrow (47-year-old female). Both nuclear and cytoplasmic staining was observed. AR expression did not show any significant deviation with age or sex. Panel b shows the expression of AR in the stromal cells (53-year-old male). AR was localized mainly in the nuclei of stromal cells (arrow-heads). Panel c illustrates the AR expression by endothelial cells lining the walls of a blood vessel (49-year-old male). Panel d shows that AR was predominantly and strongly expressed in neutrophils (24-year-old male). Both banded neutrophils (arrow-head) and segmented neutrophils (double arrow) expressed nuclear and cytoplasmic AR. In contrast, the nucleated red blood cells (arrow) did not express the receptor. Panel e shows the AR expression in metamyelocytes (7-year-old male). Perinuclear dot staining was observed (arrow-heads). In panel f, AR was not expressed in enucleated red blood cells (arrow-head). Megakaryocytes (arrow) stained weakly for AR (7-year-old male) exhibiting perinuclear dot positivity. Panel g shows that eosinophils (arrow-head) and enucleated red blood cells (arrow) did not express the receptor (24-year-old male). In panel h, AR was not expressed in lymphocytes (arrow), as shown in a benign lymphoid aggregate. The presence of a blood vessel (arrow-head) is denoted by AR staining (47-year-old female).

Clinical and experimental evidence supports a role of steroid hormones in modulating immune function. The presence of AR in thymocytes and the well-described

effects of exogenous androgens on the thymus size suggest a role of androgenic hormones in thymocyte growth and maturation [23]. Similarly, castration of

Table 1. Summary of AR expression in the bone marrow mononuclear cells

Cell type	AR positivity ¹
Myeloid	
Myeloblasts	+
Myelocytes	+
Band neutrophils	++
Segmented neutrophils	++
Basophils	nd
Eosinophils	-
Macrophages	+
Megakaryocytes	+
Lymphoid	-
Erythroid	
Mature enucleated	-
Nucleated	-
Stromal cells	+
Endothelial cells	+

¹ + indicates staining; ++ indicates strong staining; - indicates the absence of staining; * indicates perinuclear dot staining; nd = not determined.

normal mice leads to splenic enlargement and expansion of the B-cell population [13], despite splenocytes not expressing AR. Our results indicate that lymphoid cells in bone marrow also do not stain for AR. Our observation agrees in general with the work of other groups. However, Viselli *et al.* have shown the presence of AR in a murine pre-B-cell line by immunoblotting [11]. This may be due to differences between species, although our results do not exclude the possibility of AR expression in immature B-lymphocytes. The expression of AR may therefore be developmentally regulated, as it has been hypothesized that androgens act on developing cells and do not target mature T- or B-lymphocytes [24]. Moreover, the AR expression in stromal cells supports the possibility of an indirect hormonal modulation of B-cell maturation.

Studies have shown that androgens inhibit and oestrogens stimulate macrophage formation *in vitro* [25]. Our observation that AR is expressed in macrophages may therefore explain the greater immune responsiveness and higher incidence of autoimmune disease in females, if one takes into account that macrophages are antigen-presenting cells.

Although there are conflicting reports on the role of androgens in erythropoiesis, the consensus is that different testosterone metabolites appear to be active in different target cells. The 5β metabolites (5β -DHT and etiocholanolone) act directly on the stem cells (including progenitors) while 5α derivatives (5α -DHT) increase erythropoietin levels [4–6,16,26]. As such, the absence of AR expression in mature red blood cells and nucleated erythroid cells, as shown in our study, is consistent with the overall scheme of androgenic action in erythropoiesis. Furthermore, several studies have indicated the presence of a 5β receptor in erythroid precursors [4,16].

Clinical observations indicate that androgens have a multitude of effects on haematopoiesis. In some patients with aplastic anaemia, after treatment with androgens, resolution of anaemia is accompanied by granulocyte count improvement [5]. Granulocytic recovery is preceded by an increase in myeloid precursor cells, CFU-GM (colony-forming unit – granulocyte, macrophage), suggesting that androgens induce the pluripotent stem cell to proliferate and increase the committed cell compartment. Furthermore, human CD34-positive cells have been reported to express AR by RT-PCR analysis [15]. These data agree with our observation that AR was predominantly and strongly expressed in neutrophils, including band and segmented neutrophils, which express both nuclear and cytoplasmic AR. In myelocytes, AR was mainly localized to the cytoplasm and showed perinuclear dot staining. Similarly, myeloblasts expressed the receptor in their cytoplasm, but without the perinuclear dot staining. The nuclear and cytoplasmic distribution of AR appears, therefore, to be differentiation-stage specific. In conclusion, our observations suggest a direct effect of androgens on myeloid progenitor and precursor cells, as well as neutrophils. In contrast, eosinophils did not express AR.

Our results show that megakaryocytes had weak perinuclear dot AR staining. These findings agree with a recent report indicating that human megakaryocytes and platelets contain the AR [15] and with clinical observations regarding accelerated platelet count recovery in women treated with androgens after cytotoxic drug treatment [5].

In summary, the data presented in this work indicate the extensive role of androgens in haematopoiesis and the need to examine not only AR expression in the disease state, but also clinicopathological correlates of androgen levels in health and disease.

Acknowledgements

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Bicalutamide Functions as an Androgen Receptor Antagonist by Assembly of a Transcriptionally Inactive Receptor*

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Prostate cancers (PCa) that relapse after androgen deprivation therapy invariably express high levels of androgen receptor (AR) and AR-regulated genes. Most do not respond to secondary hormonal therapies, including AR antagonists, and the mechanisms of AR activation in these clinically androgen-independent tumors are unclear. Bicalutamide, the most widely used AR antagonist, is a competitive antagonist shown previously to stabilize AR association with cytosolic heat shock protein complexes. This study found nuclear AR expression in bicalutamide-treated androgen-independent PCa and found that bicalutamide could stimulate AR nuclear translocation. Moreover, specific DNA binding by the bicalutamide-liganded AR was demonstrated *in vivo* using a VP16-AR fusion protein and was confirmed by chromatin immunoprecipitation showing binding to the prostate-specific antigen enhancer in LN-CaP PCa cells. Nonetheless, bicalutamide could not stimulate interactions between the AR N and C termini or recruitment of steroid receptor coactivator proteins (SRC-1 or -2), although SRC transfection augmented AR activity in the presence of dihydrotestosterone and inhibitory concentrations of bicalutamide. These results demonstrate that bicalutamide stimulates the assembly of a transcriptionally inactive AR on DNA and support altered coactivator (or corepressor) expression as a mechanism of bicalutamide-resistant androgen-independent PCa.

The majority of prostate cancers (PCa)¹ are androgen-dependent and respond to androgen deprivation therapies, which include orchiectomy or administration of leutinizing hormone-releasing hormone agonists to suppress testicular androgen production (1). The effects of androgens on the normal prostate and PCa are mediated by binding to the androgen receptor (AR), a steroid hormone receptor member of the larger nuclear

receptor superfamily (2). Unfortunately, patients receiving androgen deprivation therapies invariably relapse with PCa that tends to be aggressive. In some cases, these relapsed tumors respond to secondary hormonal therapy or chemotherapy, but these responses are generally partial and transient, and no current therapies have been shown to prolong survival at this stage. Therefore, these prostate cancers that relapse after androgen deprivation therapy have been termed hormone refractory or androgen-independent and represent a major clinical challenge in PCa (3–5).

Although these relapsed cancers are clinically androgen-independent, many lines of evidence indicate that the AR remains active and may contribute to their androgen-independent growth. Immunohistochemical data show that the AR is highly expressed by most androgen-independent PCa (6, 7). Moreover, the AR appears to be transcriptionally active as most androgen-independent tumors express AR-regulated genes such as prostate-specific antigen (PSA). Consistent with these observations, AR mRNA appears to be increased in androgen-independent PCa, and the AR gene is amplified in ~30% of cases (8, 9). These findings have suggested that AR expression is increased in response to androgen deprivation therapy to enhance activation by residual androgens, in particular weak adrenal-derived androgens that can be converted to testosterone and dihydrotestosterone (DHT). Consistent with this hypothesis, some androgen-independent PCa patients respond to secondary hormonal agents that antagonize adrenal hormone production or to AR antagonists that directly block androgen binding to the AR (10, 11). However, these responses to further AR blockade in androgen-independent PCa occur in a minority of patients and are generally transient.

An alternative mechanism proposed to explain AR activity in androgen-independent PCa is AR mutations that result in constitutive activity or enhanced responses to other hormones. Several groups have found such mutant ARs with altered ligand responses, but their frequency in patients treated with androgen ablation monotherapy appears to be low (8, 12–14). In contrast, AR mutations were found in ~40% of patients who relapsed after initial combined therapy with the AR antagonist flutamide (15). Moreover, these mutations resulted in mutant ARs that were strongly activated by flutamide and other steroid hormones, indicating selective pressure to maintain AR activity. This flutamide-treated patient population also had an increased response rate to secondary hormonal therapy with bicalutamide, an AR antagonist that remains effective against identified AR mutants (11). However, responses were generally partial and transient, further indicating a limited overall role for AR mutations as a mechanism for AR activity in androgen-independent PCa.

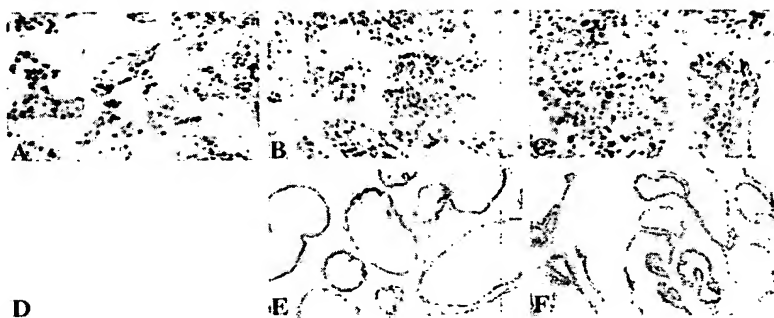
Based on these observations, mechanisms proposed to account for AR activity in androgen-independent PCa should be

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¹ The abbreviations used are: PCa, prostate cancer(s); AR, androgen receptor; hAR, human AR; ARE, androgen-responsive element; ARN, N-terminal AR; SRC, steroid receptor coactivator; PSA, prostate-specific antigen; CS-FCS, charcoal-dextran-stripped fetal calf serum; DHT, dihydrotestosterone; PBS, phosphate-buffered saline; Luc, luciferase.

FIG. 1. Nuclear AR expression in bicalutamide-treated PCa and normal mouse prostate. A–C, formalin-fixed and paraffin-embedded bone marrow biopsy sections from bicalutamide-treated androgen-independent PCa were immunostained with anti-AR antibody. D, normal mouse prostate immunostained with nonimmune antisera. E and F, anti-AR immunostained prostates from untreated (E) or bicalutamide-treated (F) mice.



consistent with and account for resistance to AR antagonists. The most widely used AR antagonists have been the steroidal drug cyproterone acetate and the nonsteroidal drugs flutamide and bicalutamide, which are all competitive antagonists of androgen binding (16). Cyproterone acetate has significant AR agonist activity, whereas weak agonist activity has been shown for hydroxyflutamide, the active metabolite of flutamide (17, 18). In contrast, previous studies have shown bicalutamide to be a pure antagonist of wild type and identified mutant ARs (18, 19). The unliganded AR associates with a heat shock protein 90 (HSP90) chaperone complex that facilitates ligand binding with subsequent conformational changes resulting in AR homodimerization, nuclear translocation, DNA binding, and transcriptional activation (20). Previous studies in cell lines have indicated that the bicalutamide-liganded AR remains cytoplasmic- and HSP90-associated, which is not consistent with transcriptional activity (21). This study further examined the effects of bicalutamide on the AR to identify mechanisms that may contribute to androgen-independent and bicalutamide-resistant AR activity in androgen-independent PCa.

EXPERIMENTAL PROCEDURES

Plasmids—A human AR expression vector, pSVARo, was from A. Brinkmann (22). pRL-CMV, pG5-Luc, pBind, and pACT vectors were from Promega (Madison, WI). VP16 activation domain-AR expression vectors were generated in pACT containing the full-length AR (pACT-hAR) or the N-terminal AR (amino acids 1–505, pACT-ARN) (23). The Gal4 DNA binding domain was fused to the AR ligand binding domain (amino acids 661–919) to generate pBind-ARLBD. The ARE₄-Luc reporter contained four tandem androgen-responsive elements cloned into pGL3 (23). Expression vectors for human SRC1 (pSG5-SRC1) and murine GRIP1 (pCMV-GRIP1) were from M. Brown (Dana-Farber Cancer Institute, Boston, MA) (24, 25).

Immunohistochemistry and Immunofluorescence—Bone marrow biopsies from patients with androgen-independent PCa being treated with bicalutamide were obtained as described (15) and were immunostained using a rabbit anti-AR N-terminal antibody (PG-21, Upstate Biotechnology, Lake Placid, NY). Mouse prostates were harvested and immunostained with the same antibody, as described (26). LNCaP cells were grown on coverslips in RPMI 1640 with 10% charcoal-dextran-stripped FCS (CS-FCS, Hyclone, Logan UT), which is steroid hormone-depleted. Cells were then treated with DHT (Sigma) or bicalutamide (provided by Astra-Zeneca, Wilmington, DE) as indicated, fixed in PBS/4% paraformaldehyde, and stained with the AR antibody and a secondary goat anti-mouse fluorescein isothiocyanate conjugate.

Transient Transfections and Reporter Gene Assays—CV1 cells were cultured in 24-well plates in Dulbecco's modified Eagle's medium with 10% FCS to ~90% confluence. Cells were transfected by mixing the indicated amounts of plasmid DNA with 1.5 μ l of LipofectAMINE 2000 (Invitrogen) in a final volume of 100 μ l of Opti-MEM for 25 min at room temperature, which was then mixed with 0.5 ml of Dulbecco's modified Eagle's medium with 10% CS-FCS and added to the cell culture wells. After 24 h, the culture medium was replaced with 0.2 ml of Dulbecco's modified Eagle's medium/10% CS-FCS with or without DHT or bicalutamide as indicated. After another 24 h, the cells were assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase reporter assay system (Promega). The results were normalized for the internal *Renilla* control and expressed as the mean relative light units plus standard deviation from triplicate or quadruplicate samples.

Flow Cytometry—The LNCaP PCa cell line was cultured in RPMI 1640/10% FCS until 70–80% confluent in 10-cm plates. They were then transferred to RPMI-160/10% CS-FCS for 24 h and treated with the indicated amount of DHT or bicalutamide for another 24 h. Cells were recovered by trypsin, washed, and resuspended in 0.5 ml of PBS/5 mM EDTA, and 0.7 ml of 95% ethanol was then added. On the day of analysis, RNase (2.5 μ l at 10 mg/ml) was added for 30 min followed by 5 μ l of an 0.5 mg/ml solution of propidium iodide.

Chromatin Immunoprecipitation—LNCaP cells grown to ~80% confluence in 10-cm plates were switched to steroid hormone-depleted medium (RPMI 1640/10% CS-FCS) for 48 h and then exposed to 10 nM DHT or 100 μ M bicalutamide for varying times. Plates were then rinsed with PBS and fixed for 10 min at room temperature with 1% formaldehyde in PBS. After rinsing twice with ice-cold PBS, cross-linking was terminated by scraping cells into 1 ml of 100 mM Tris, pH 9.4, and 10 mM dithiothreitol and incubating at 30 °C for 15 min. Cell pellets were then washed twice with PBS and resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors). Chromatin was sheared to 300–1000 bp with three sequential 10-s pulses at 70% power with a microtip ultrasonic cell dismembrator (Fisher). Cell debris was pelleted at 10,000 \times g, and the supernatant was precleared with 10 μ g of sheared salmon sperm DNA, 20 μ l of nonimmune sera, and 20 μ l of a 50% slurry of protein A-Sepharose. Immunoprecipitation was with 500 ng of AR N-terminal rabbit polyclonal antibody (PG-21, Upstate Biotechnology), 2 μ g of sheared salmon sperm DNA, and 20 μ l of a 50% slurry of protein A-Sepharose. Control immunoprecipitations were with 500 ng of a rabbit anti-platelet-derived growth factor receptor antibody (Upstate Biotechnology). Precipitates were washed three times with 300 mM NaCl, 50 mM Tris, pH 8.0, 2.7 mM KCl, 0.05% Tween 20, and 1% deoxycholate. Three additional washes with 10 mM Tris, 1 mM EDTA were performed, and beads were then eluted three times with 35 μ l of 1% SDS and 100 mM NaHCO₃ at 37 °C for 10 min each. Eluates were pooled and incubated at 65 °C overnight to reverse cross-links. Products were then purified with QIAquick PCR purification spin resin (Qiagen, Valencia, CA), and 10% of the eluate was subjected to 50 cycles of PCR amplification with steps of 95, 55, and 72 °C for 1 min each. The primers were TGAGAAACCT-GAGATTAGGA and ATCTCTCTCAGATCCAGGCT, corresponding to nucleotides –4270 to –4250 and –4065 to –4045, respectively, in the PSA enhancer, which flank the major androgen-responsive element (ARE) regulating this gene (27–29). PCR products were analyzed by gel electrophoresis and ethidium bromide staining in 5% agarose gels. Inputs represent 5% of the DNA used for the AR immunoprecipitations, which was similarly treated to reverse cross-links and then PCR-amplified using the same PSA primers.

RESULTS

Bicalutamide Does Not Prevent AR Nuclear Localization in Prostate Cells—Previous biochemical studies showed that bicalutamide can maintain the AR in a cytoplasmic complex with HSP90 (21). Immunohistochemical studies of the AR in androgen-independent PCa have generally shown strong nuclear expression, but AR in bicalutamide-treated patients with androgen-independent PCa has not been specifically examined. Therefore, tumor-containing bone marrow biopsies obtained from bicalutamide-treated patients with androgen-independent PCa were examined for AR expression. The results demonstrated strong nuclear AR expression in each of four cases examined (Fig. 1, A–C, and data not shown).

To determine whether nuclear localization was inhibited by

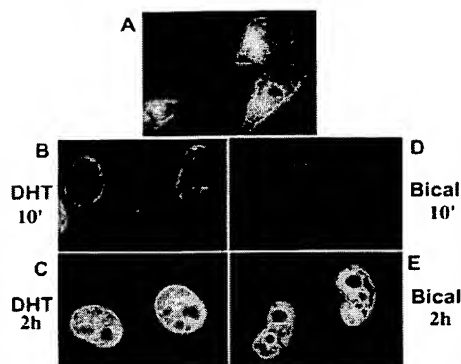


FIG. 2. Bicalutamide (Bical)-stimulated AR nuclear localization in LNCaP cells. LNCaP cells were grown on coverslips in steroid hormone-depleted medium (A) and then stimulated for 10 min (B and D) or 2 h (C and E) with 10 nM DHT (B and C) or 10 μ M bicalutamide (D and E). Cells were then fixed, and AR expression was analyzed by indirect immunofluorescence.

bicalutamide in normal prostate epithelium, mice were treated with bicalutamide (1 mg in 0.1 ml of PBS by intraperitoneal injection every other day for 2 weeks), and their prostates were examined. The seminal vesicles in the treated mice were markedly shrunk, indicative of AR inhibition (not shown). However, immunohistochemistry showed strong nuclear AR expression in the prostate epithelium of both untreated and bicalutamide-treated mice (Fig. 1, E and F). These results demonstrated that bicalutamide did not prevent AR nuclear translocation in normal or malignant prostate epithelial cells.

To assess the effect of bicalutamide on AR cellular localization in PCa cells *in vitro*, the LNCaP prostate cancer cell line was examined. LNCaP expresses a mutant AR that is activated by 8-hydroxyflutamide (the active metabolite of flutamide) but is still blocked by bicalutamide (19, 30). Moreover, bicalutamide can inhibit LNCaP growth and PSA production. Consistent with previous studies, immunofluorescence showed that the AR in LNCaP cells grown in steroid hormone-depleted medium was diffusely distributed in the cytoplasm and nucleus (Fig. 2A). Treatment with DHT resulted in rapid nuclear accumulation with the AR being almost completely nuclear at 2 h (Fig. 2, B and C). Treatment with bicalutamide also resulted in rapid nuclear translocation (Fig. 2, D and E). Similar results have been obtained using transfected wild type AR in HeLa cells (data not shown). Taken together, these findings indicated that bicalutamide, in addition to not blocking nuclear expression of AR in prostate epithelium, had agonist activity with respect to AR nuclear translocation.

Bicalutamide Can Stimulate AR DNA Binding—Despite nuclear AR expression, bicalutamide can completely block DHT-stimulated AR transactivation and has no detectable partial agonist activity on the full-length AR (18). However, transcriptional activation by the full-length AR is a multistep process involving DNA binding, homodimerization, interaction between the AR N and C termini, and association with coactivator proteins. Therefore, to assess bicalutamide effects on DNA binding independent of these latter protein-protein interactions, the heterologous VP16 transactivation domain was fused to the N terminus of full-length AR. The VP16-AR (2–919) fusion protein was inactive in the absence of androgen but could be strongly stimulated by DHT (Fig. 3A, left panel). Bicalutamide similarly induced substantial activation of the VP16-AR (2–919) fusion (Fig. 3A, right panel). Importantly, maximal activity was seen in the micromolar concentration range, well within the bicalutamide levels in patients treated with this drug (31). Therefore, this result indicated that bicalu-

tamide had partial agonist activity with respect to stimulating AR binding to DNA.

Chromatin immunoprecipitation was used to further assess whether bicalutamide stimulated AR binding to an endogenous androgen-regulated gene. LNCaP or DU145 (AR-negative) PCa cells grown in steroid hormone-depleted medium were treated with DHT or bicalutamide. They were then examined by chromatin immunoprecipitation for AR binding to the PSA enhancer (–4270 to –4045 from the start site), which contains the major ARE regulating this gene and multiple weak AREs (27–29). Immunoprecipitates with an irrelevant antibody (anti-platelet-derived growth factor receptor) and AR immunoprecipitates from the DU145 cells or the untreated LNCaP cells contained little or no detectable PSA enhancer DNA (Fig. 3B). In contrast, both DHT and bicalutamide treatments stimulated AR association with the enhancer, further supporting the conclusion that bicalutamide stimulates AR binding to AREs.

Bicalutamide Does Not Support Association of the AR N and C Termini or Interaction with Steroid Receptor Coactivator Proteins—Previous studies showed that a ligand-dependent interaction between the AR N and C termini made a major contribution to AR transcriptional activity and that this interaction was not stimulated by antagonists including cyproterone acetate or flutamide (32–34). A mammalian two-hybrid protein binding assay was similarly used to determine whether bicalutamide could stimulate this interaction. Cells were transfected with vectors encoding VP16-AR (2–505) and Gal4-AR (661–919) fusion proteins (corresponding to the AR N-terminal and ligand binding domains, respectively) in conjunction with a luciferase reporter gene regulated by tandem Gal4-responsive elements (pG5-Luc). There was no detectable interaction between these proteins in the absence of ligand, but the interaction could be strongly stimulated by DHT (Fig. 4, left panel). In contrast, bicalutamide did not stimulate this interaction.

The transcriptional activity of steroid hormone receptors is also mediated by ligand-dependent binding of coactivator proteins, in particular SRC-1 and -2 (35), and increased expression of these coactivators has been reported in androgen-independent PCa (36, 37). These coactivator proteins have leucine-X-X-leucine-leucine (LXXLL) motifs that mediate ligand-dependent binding to the ligand binding domain and contain a distinct site that binds to the N-terminal, with binding to the AR being mediated primarily by this latter N-terminal interaction (38, 39). To determine whether the bicalutamide-liganded AR could interact with SRC-1, cells were cotransfected with AR and SRC-1 expression vectors. Consistent with previous reports, SRC-1 could augment DHT-stimulated AR transcriptional activity (Fig. 5A). However, SRC-1 did not stimulate AR activity in the absence of ligand or in the presence of bicalutamide. SRC-2 transfection similarly enhanced DHT-stimulated transcriptional activity (although the effect was more modest) but not ligand-independent or bicalutamide-mediated activity (Fig. 5B). These results indicated that unliganded or bicalutamide-liganded AR could not associate with these coactivators, which did not support SRC-1 or SRC-2 overexpression as a mechanism of AR activity in androgen-independent PCa.

Although the SRC proteins did not stimulate activity of the unliganded or bicalutamide-liganded AR, castrated males still produce androgens that can be converted to DHT in prostate cells. Therefore, further experiments addressed whether SRC proteins could enhance AR responses to lower levels of DHT. Dose response studies showed that SRC-1 transfection enhanced DHT-stimulated AR activity over a broad range of DHT concentrations (Fig. 5, C and D). However, a marked left shift

FIG. 3. DNA binding by bicalutamide (*Bical*)-liganded AR. In A, CV1 cells were transfected with 100 ng of pACT-hAR, encoding a VP16-AR (2-919) fusion protein, 250 ng of ARE₃-Luc reporter, and 0.2 ng of pRL-CMV control (A). At 24 h after transfection, cells were treated for 24 h with the indicated concentrations of DHT or bicalutamide, and firefly *versus* Renilla luciferase was measured. RLU, relative light units. In B, DU145 or LNCaP cells grown in steroid hormone-depleted medium were stimulated for 15 or 30 min with 10 nM DHT or 100 μ M bicalutamide (*BIC*) followed by formalin cross-linking, chromatin immunoprecipitation with anti-AR antibody, and PCR amplification to detect the PSA enhancer ARE. IP, immunoprecipitates.

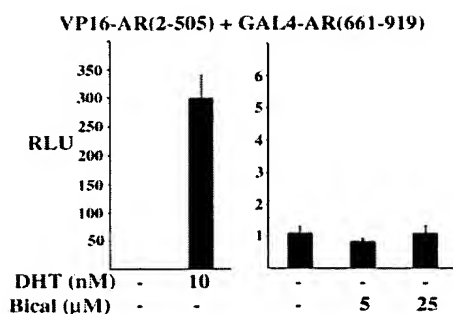
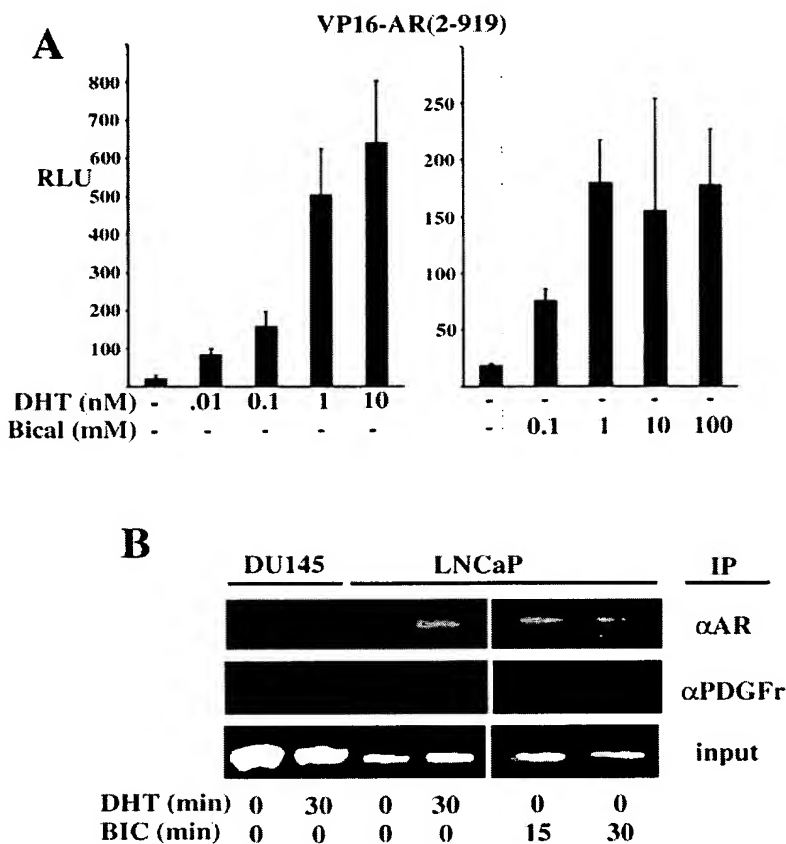


FIG. 4. Lack of interaction between the AR N terminus and bicalutamide (*Bical*)-liganded C terminus. CV1 cells were transfected with pACT-ARN, encoding a VP16-AR (2-505) fusion protein, pBind-ARLBD, encoding a Gal4DBD-AR (661-919) fusion protein, a pG5-Luc reporter, and a control pRL-CMV vector. After transfection, cells were treated for 24 h with DHT or bicalutamide as indicated. RLU, relative light units.

in the dose response curve to lower DHT concentrations was not observed, suggesting that SRC-1 did not increase AR affinity for DHT.

Coactivator Expression Increases the Bicalutamide Concentrations Required to Antagonize AR Transcriptional Activity—It was next determined whether SRC-1 overexpression diminished the ability of bicalutamide, at concentrations obtained *in vivo*, to inhibit DHT-stimulated AR transcriptional activity. As shown in Fig. 6A, bicalutamide at 5 μ M could completely block the AR transcriptional activity stimulated by 10 nM DHT. In contrast, SRC-1-transfected cells treated with 10 nM DHT and 5 μ M bicalutamide had substantial AR activity with lower but

still detectable activity at 25 μ M bicalutamide. Cotransfection with SRC-2 similarly stimulated AR transcriptional activity in the presence of DHT and 1–5 μ M bicalutamide (Fig. 6B). Further dose response studies showed that SRC-1 transfection did not markedly alter the IC₅₀ for bicalutamide, which was between 0.1 and 1 μ M (data not shown). In conjunction with the above DHT dose response studies, these results indicated that SRC-1 was functioning primarily by augmenting the transcriptional activity of agonist-bound AR rather than by decreasing the ability of bicalutamide to compete with DHT for AR binding.

Effects of Bicalutamide Dose Escalation on LNCaP Cells—A prediction based on the above data was that androgen-independent PCa cells overexpressing SRC proteins should respond to higher doses of bicalutamide or to more potent AR antagonists. Such AR-expressing cell lines derived from androgen-independent PCa are not available, but the LNCaP prostate cancer cell line has features of androgen-independent PCa. In particular, although LNCaP proliferation and PSA production are stimulated by DHT, they are only partially inhibited by bicalutamide at 5–10 μ M. Therefore, the effects of higher bicalutamide concentrations on these cells were assessed.

PSA production by LNCaP cells was inhibited progressively by bicalutamide concentrations up to 100 μ M (Fig. 7A). Inhibition of LNCaP cell growth as assessed by the percentage of cells in S phase was also progressively inhibited at bicalutamide concentrations up to 40–80 μ M (Fig. 7B). The inhibitory effects of bicalutamide were abrogated by added DHT, indicating that the inhibition reflected AR blockade and not nonspecific toxicity due to the high bicalutamide concentrations (Fig. 7C). These findings indicated that the AR in LNCaP cells remained active

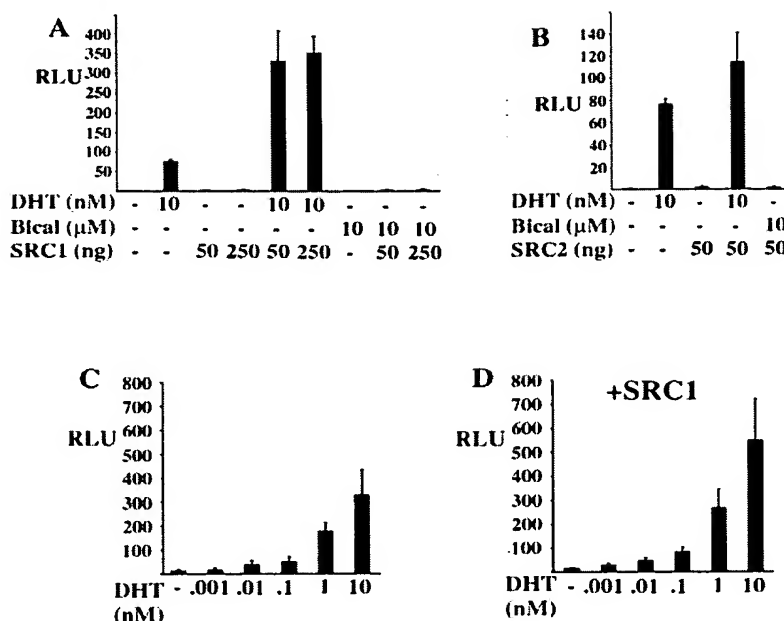


FIG. 5. SRC fails to stimulate bicalutamide (*Bical*)-liganded AR. In A and B, CV1 cells were cotransfected with AR (pSVARo), ARE₄-Luc, control pRL-CMV, and the indicated amounts of SRC1 (A) or SRC2 (B) expression vectors. After transfection, cells were treated with DHT or bicalutamide as indicated for 24 h and harvested. *RLU*, relative light units. In C and D, cells were transfected as above without (C) or with (D) SRC1 expression vector.

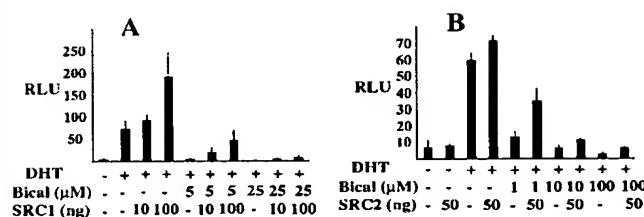


FIG. 6. SRC stimulation of AR activity in cells treated with DHT and bicalutamide (*Bical*). In A and B, CV1 cells were cotransfected with AR (pSVARo), ARE₁-Luc, control pRL-CMV, and the indicated amounts of SRC1 (A) or SRC2 (B) expression vectors and were then treated with hormones as indicated. *RLU*, relative light units.

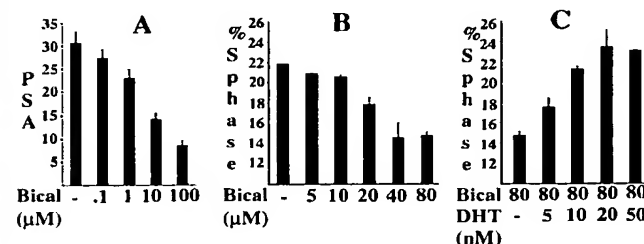


FIG. 7. Inhibition of LNCaP PSA production and proliferation by bicalutamide (Bical). In A, LNCaP cells were grown in complete medium with 10% FCS and the indicated concentrations of bicalutamide for 72 h, and PSA secretion (ng/ml) into the culture supernatant was measured by enzyme-linked immunosorbent assay during the last 24 h. RLU, relative light units. In B and C, LNCaP cells were grown in complete medium with 10% FCS and treated for 48 h with bicalutamide and DHT as indicated. The percentage of S phase was then assessed by propidium iodide staining and flow cytometry.

even at 10 μM bicalutamide and could be antagonized by higher concentrations of the drug.

DISCUSSION

Previous reports have suggested that bicalutamide functioned as a pure antagonist by preventing AR DNA binding (18–21). In contrast, the data reported here demonstrate that the bicalutamide-liganded AR localizes in the nucleus and,

more importantly, that it can bind to AREs in episomal reporter genes and in the endogenous PSA promoter. However, the bicalutamide-liganded AR is transcriptionally inactive. Consistent with this lack of transcriptional activity, bicalutamide did not stimulate binding of the AR N terminus to the C-terminal ligand binding domain. Moreover, cotransfection of bicalutamide-treated cells with SRC-1 or -2 did not stimulate AR activity, indicating that these coactivator proteins did not interact with the bicalutamide-liganded AR. Taken together, these findings indicate that the bicalutamide-liganded AR binds to DNA but in a transcriptionally inactive conformation.

The lack of AR N- and C-terminal interaction in the bicalutamide-liganded AR is not surprising as this is mediated by an LXXLL-like motif in the N terminus and is dependent on an agonist conformation in the ligand binding domain (40). However, SRC-1 binding to the AR is primarily or exclusively through the N terminus as the LXXLL motifs in SRC-1 do not bind to the AR ligand binding domain (39, 41). The precise site in the AR N terminus that mediates SRC-1 binding has not been identified, but the lack of SRC-1 interaction with the bicalutamide-liganded AR indicates that the N-terminal binding site may be affected by ligand binding, possibly reflecting a role for the AR N- and C-terminal interaction in positioning or exposing the N-terminal SRC-1 binding site. Alternatively, binding of a corepressor to the bicalutamide-liganded AR could block SRC-1 binding or inhibit its coactivator functions (see below).

Although SRC-1 and -2 did not stimulate transcriptional activation of the unliganded or bicalutamide-liganded AR, higher concentrations of bicalutamide were needed to block DHT-stimulated transcriptional activity in cells transfected with SRC-1 or -2. This did not appear to reflect an increase in the affinity for DHT relative to bicalutamide as SRC-1 transfection did not markedly alter the dose response to DHT or the apparent IC₅₀ for bicalutamide inhibition of DHT-stimulated AR activity. The most straightforward explanation for these results is that SRC-1 and -2 are rate-limiting coactivators and enhance the transcriptional activity of the small fraction of DHT-liganded ARs. Importantly, this enhancement could be relevant in PCA patients being treated with androgen deprivation.

tion therapy (castration or luteinizing hormone releasing hormone agonist) in conjunction with bicalutamide. In this setting, in which there are still substantial levels of circulating androgens, increased expression of SRC proteins would diminish the efficacy of bicalutamide in blocking residual AR activity.

Recent reports demonstrate that the AR can also bind to corepressor proteins, including nuclear corepressor (NCoR), indicating that corepressor binding could further contribute to the *in vivo* antagonist activity of bicalutamide (42–44). This would suggest down-regulation of a corepressor as another mechanism for development of bicalutamide-resistant PCA. These data also suggest that bicalutamide may function as a selective AR modulator. However, in contrast to the selective estrogen receptor modulators tamoxifen and raloxifene, bicalutamide does not appear to activate the N-terminal activation function (AF-1) of the AR, and cell types or tissues in which bicalutamide functions as an AR agonist have not been identified.

In summary, these results demonstrate that bicalutamide does not function as an AR antagonist by preventing AR binding to DNA but instead stimulates the assembly of a transcriptionally inactive receptor on DNA. These findings support a continued role for the AR in androgen-independent bicalutamide-resistant PCA and suggest that the increased expression of AR and transcriptional coactivator proteins in androgen-independent PCA may decrease the efficacy of bicalutamide in blocking AR activation by residual androgens. Importantly, this mechanism predicts that inhibition of adrenal androgen production in conjunction with bicalutamide (or more potent AR antagonists) may have efficacy in androgen-independent PCA.

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Effects of Dihydrotestosterone on Bone Biochemical Markers in Sham and Oophorectomized Rats

R.A. MASON and H.A. MORRIS

ABSTRACT

Evidence exists to suggest that androgens stimulate bone formation in the estrogen-deficient state, however the mechanism of action is unclear. The following study investigates the effect of dihydrotestosterone (DHT) on biochemical markers of bone turnover and calcium homeostasis in sham and oophorectomized (oophx) rats when either vehicle, 40, 80, or 160 mg/kg body weight (bw) DHT were administered at the time of operation or at 15 weeks postoperation. Serum alkaline phosphatase (ALP) increased following DHT administration in sham and oophx rats in all groups (mean ALP \pm SEM [U/l] week 8; sham vehicle, 40 ± 7 ; sham 160 mg DHT/kg bw, 72 ± 5 ; oophx vehicle, 60 ± 6 ; oophx 160 mg DHT/kg bw, 88 ± 11) ($p < 0.001$). In contrast, serum osteocalcin was significantly suppressed in oophx rats administered DHT 15 weeks following operation (mean osteocalcin \pm SEM [μ g/l] week 8; oophx vehicle, 17.6 ± 3.5 ; oophx 160 mg DHT/kg bw, 10.5 ± 1) ($p < 0.01$). Urine deoxypyridinoline was significantly decreased when DHT was administered 15 weeks post-oophorectomy ($p < 0.001$); however, urine hydroxyproline was not affected by DHT treatment in any group. Urine calcium was decreased by DHT treatment (mean Ca/Cr \pm SEM week 8; sham vehicle, 0.87 ± 0.13 ; sham 160 mg DHT/kg bw, 0.24 ± 0.08 ; oophx vehicle, 0.68 ± 0.16 ; oophx 160 mg DHT/kg bw, 0.45 ± 0.1) ($p < 0.005$) which was associated with an increase in the renal tubular reabsorption of calcium ($p < 0.05$). This study demonstrates the direct effects of DHT on both bone cell activities and the renal handling of calcium. (J Bone Miner Res 1997;12:1431-1437)

INTRODUCTION

ANDROGENS PARTIALLY RESTORE bone in both postmenopausal women and gonadectomized male and female rats.⁽¹⁻⁸⁾ While androgen receptors have been identified on human osteoblast-like cells,⁽⁹⁾ it is unclear whether these responses are an effect of androgens on bone cells to stimulate bone formation or inhibit bone resorption. Alternatively, they may exert an indirect effect on bone by increasing muscle mass and thereby stimulating mechanical forces on the skeleton. Some androgens may be converted within the target cell to estrogen and thus effects may also be exerted via activation of the estrogen receptor.

In postmenopausal osteoporotic women, the synthetic androgen nandrolone decanoate increases forearm mineral density and calcium absorption with no effect observed on the biochemical marker of bone resorption, urine hy-

droxyproline excretion.⁽⁴⁾ Such data suggest that androgens act to increase bone mineral density by increasing bone formation. In contrast, Johansson and coworkers found nandrolone combined with oral calcium supplementation, also in postmenopausal osteoporotic women, increased bone mineral content with no concomitant rise in bone formation. They suggested that androgens have a positive effect on bone by inhibiting bone resorption in addition to increasing muscle mass.⁽¹⁾

The oophorectomized (oophx) rat has been used extensively as a model of postmenopausal bone loss.⁽¹⁰⁾ Adrenalectomized female rats suffer bone loss similar to the oophx rat which is reversed by treatment with nandrolone decanoate.⁽¹¹⁾ 5 α -dihydrotestosterone (DHT) partially restores cancellous bone volume in osteopenic oophx rats, reflecting a net gain of bone tissue rather than the prevention of further bone loss.^(5,8) Furthermore, in female rats, the ad-

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ministration of the androgen antagonist flutamide induces bone loss independent of estrogen status.⁽¹²⁾

Such data demonstrate the importance of androgens for skeletal integrity in females as well as males. The mechanism of androgens on bone, however, remains controversial and to differentiate between an antiresorptive action and stimulation of osteoblast activity we have investigated the effects of DHT on biochemical indices of bone turnover and calcium homeostasis in sham and oophx rats. Unlike testosterone, DHT is not metabolized to estrogen and therefore the effects observed following such treatment are due to activation of the androgen receptor.

MATERIALS AND METHODS

Animals

Seventy-two 8-month-old female Sprague-Dawley rats (265–357 g) were obtained from the Gilles Plains Animal Resource Centre (Gilles Plains, South Australia). Each rat was meal fed 20 g/day commercial rat chow containing 0.7% calcium and 0.6% phosphorus and 200 U/kg vitamin D (Milling Industries Pty. Ltd., South Australia) and tap water was supplied ad libitum. The animals were housed at 26°C with a 12-h light–dark cycle. All procedures were approved by The Institute of Medical and Veterinary Science and The University of Adelaide animal ethic committees.

Experimental procedure and design

Experiment 1: Forty-eight animals were randomly divided into eight groups, and preoperative fasting blood and urine samples were collected to provide baseline biochemistry. The rats were randomly allocated to either a sham or oophorectomy operation performed under halothane anaesthesia and were administered either vehicle (silastic tubing), 40, 80, or 160 mg/kg body weight (bw) DHT (Sigma Chemical Co., Milwaukee, WI, U.S.A.) by silastic implants (Dow-Corning Medical Silastic tubing, Dow-Corning, Midland, MI, U.S.A.) of lengths 1.5, 2.8, and 5.6 cm, respectively, at the time of operation. Twenty-four hour fasting blood and urine samples were collected weekly for 8 weeks.

Experiment 2: Twenty-four animals were randomly divided into four groups, and all animals were oophorectomized under halothane anaesthesia. At 15 weeks postoperation, the rats were administered either vehicle (silastic tubing), 40, 80, or 160 mg/kg (bw) DHT as for experiment 1. Twenty-four hour fasting blood and urine samples were collected every 2 weeks for 14 weeks.

Biochemical analyses

Urine volumes were recorded. Urine creatinine, acidified urine calcium, serum alkaline phosphatase, serum alanine aminotransferase, serum calcium, albumin, total protein, and creatinine were analyzed on automated chemical analysers (Kone Progress Plus, Kone Corp., Ruukintie, Finland and Cobas Bio, Roche Ltd., Basel, Switzerland) using manufacturer recommended methods. Serum sodium, potassium, chloride, and bicarbonate were measured on a Fast 4

electrolyte system (Ciba-Corning 664, Medfield, MA, U.S.A.). Whole blood ionized calcium was measured on a calcium-pH analyzer (Ciba-Corning 634, Halstead Essex, U.K.). Urine hydroxyproline was measured by the method of Bergman and Loxley.⁽¹³⁾ Free urine deoxypyridinoline was measured by competitive enzyme immunoassay using a commercially available kit (Pyrilinks-D, Metra Biosystems Inc., CA, U.S.A.). Serum osteocalcin was measured by radioimmunoassay as described by Morris et al.⁽¹⁴⁾ DHT was measured by radioimmunoassay using a commercially available kit (Diagnostic System Laboratories Inc., Webster, TX, U.S.A.).

Calculations and statistical analyses

Urine calcium, hydroxyproline, and free deoxypyridinoline were expressed as a ratio to urine creatinine. Creatinine excretion (mmol/day) was calculated by multiplying urine creatinine (mmol/l) by 24-h urine volume (l/day). Ultrafiltrable calcium was calculated by the formula described by Morris et al.⁽¹⁴⁾ Anion gap was calculated from the difference between the sum of the serum sodium and potassium and the sum of the serum chloride and bicarbonate. The maximum tubular reabsorption of calcium (TmCa) was calculated by the formulas described by Marshall⁽¹⁵⁾; however, the calculated serum ultrafiltrable calcium was used as the measure of the filtered load of plasma calcium. Statistical analyses for hydroxyproline/creatinine ratio, alkaline phosphatase, and osteocalcin were performed on the difference between the experimental values and the baseline value to correct for individual variation.

One way analysis of variance (ANOVA) was used to analyze the effect of operation in rats receiving vehicle alone. Multiple comparisons of mean values were made by repeated measures ANOVA. When the assumption of equal correlations between biochemical analyses at each time point was violated, a conservative adjustment to the degrees of freedom was made using the following formula⁽¹⁶⁾:

degrees of freedom
(adjusted)

$$= \frac{\text{within subject degrees of freedom}}{(\text{number of within subject groups} - 1)}$$

Tukey's post hoc test was used to identify significant differences between mean values.⁽¹⁷⁾ All data were analyzed by SAS version 6.10 (SAS Institute, Cary, NC, U.S.A.) on a personal computer. A value of $p < 0.05$ was considered significant.

RESULTS

Serum DHT levels, which were unaffected by operation, correlated with dose administered in both sham and oophx rats ($p < 0.001$), and remained elevated throughout the duration of both experiments (Table 1). DHT administration did not significantly affect urine volume or 24-h urine creatinine excretion in either experiment (data not present-

TABLE 1. SERUM DIHYDROTESTOSTERONE LEVELS (pmol/l) MEASURED IN EXPERIMENT 1 AND 2

Time (weeks)	Control				Oophx			
	Vehicle	40 mg	80 mg	160 mg	Vehicle	40 mg	80 mg	160 mg
2	—	2143 (423)*†	2253 (289)*†	5060 (1241)*††	—	1338 (334)*†	2394 (602)*†	5772 (1073)*††
5	—	1307 (251)*	1483 (244)*	4355 (1118)*†	—	1355 (575)*	1864 (530)*	4372 (482)*†
8	231 (38)	1335 (237)*	1369 (234)*	3922 (626)*†	158 (21)	1011 (210)*	1882 (695)*	3570 (488)*†
14	—	—	—	—	172 (24)	1022 (148)*	1352 (186)*	2580 (451)*†

Values are mean (SE). * $p < 0.05$ versus vehicle within operation group; † $p < 0.05$ versus 40 and 80 mg DHT; †† $p < 0.05$ versus weeks 8 and 14 within dosage group.

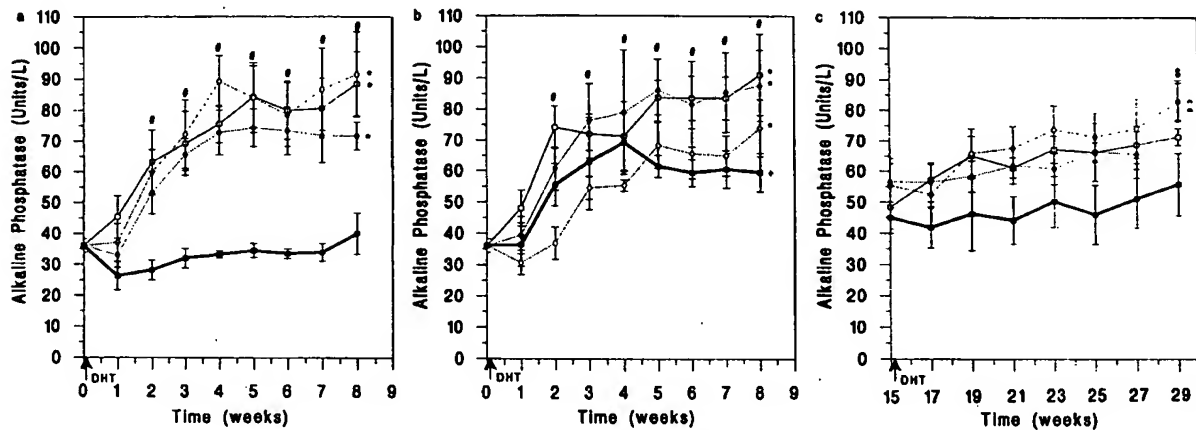


FIG. 1. Changes in serum alkaline phosphatase (U/l) in sham (a) and oophx (b) rats administered DHT from the time of operation and in oophx (c) rats administered DHT between 15 and 29 weeks following oophorectomy. (■) Vehicle, (□) 40 mg DHT, (◇) 80 mg DHT, (◆) 160 mg DHT. * $p < 0.001$ versus sham (a) within dosage group; † $p < 0.01$ versus vehicle within operation group; †† $p < 0.0001$ versus week 1; § $p < 0.001$ versus weeks 15 and 17; ^ $p < 0.05$ versus vehicle within operation group.

ed), and urine biochemical variables were expressed as a ratio to creatinine to correct for dilutional and bladder emptying errors.

Experiment 1

Serum alkaline phosphatase and osteocalcin were raised as a result of oophorectomy in rats receiving vehicle ($p < 0.001$) (Figs. 1a, 1b, 2a, and 2b). Alkaline phosphatase was elevated following DHT administration independent of dose ($p < 0.001$) in both sham and oophx rats. This effect was time dependent ($p < 0.001$) with maximal stimulation occurring between weeks 2 and 8 after commencing treatment (Figs. 1a and 1b). Osteocalcin was unaffected by DHT treatment in either operation group (Figs. 2a and 2b). There was no difference between the sham and oophx rats following DHT treatment due to an insignificant rise in the sham rats and an insignificant fall in the oophx rats.

Urine hydroxyproline/creatinine and deoxypyridinoline/creatinine were increased as a result of oophorectomy in rats receiving vehicle ($p < 0.001$, $p < 0.01$, respectively)

(Table 2); however, they were unaffected by DHT administration in either group. Although not statistically significant, DHT treatment resulted in an increase in both urine hydroxyproline and deoxypyridinoline in sham rats such that no statistical difference was observed between DHT-treated sham and oophx rats (Table 2). Urine calcium/creatinine was unaffected by operation in rats receiving vehicle (Figs. 3a and 3b). DHT administration decreased urine calcium independent of dose ($p < 0.005$) in both sham and oophx rats. This effect was time dependent and occurred at 2 weeks following commencement of DHT treatment ($p < 0.005$) (Figs. 3a and 3b). TmCa was unaffected by oophorectomy but was increased following DHT administration ($p < 0.05$) (Table 2) and this effect was not dose dependent but was time dependent with maximal responses occurring at weeks 6–8, following DHT administration (data not presented). Ionized calcium was not affected by oophorectomy but was significantly decreased at all doses of DHT treatment ($p < 0.05$), (Table 2) with maximal suppression occurring at weeks 6–8 following commencement of DHT administration (data not presented).

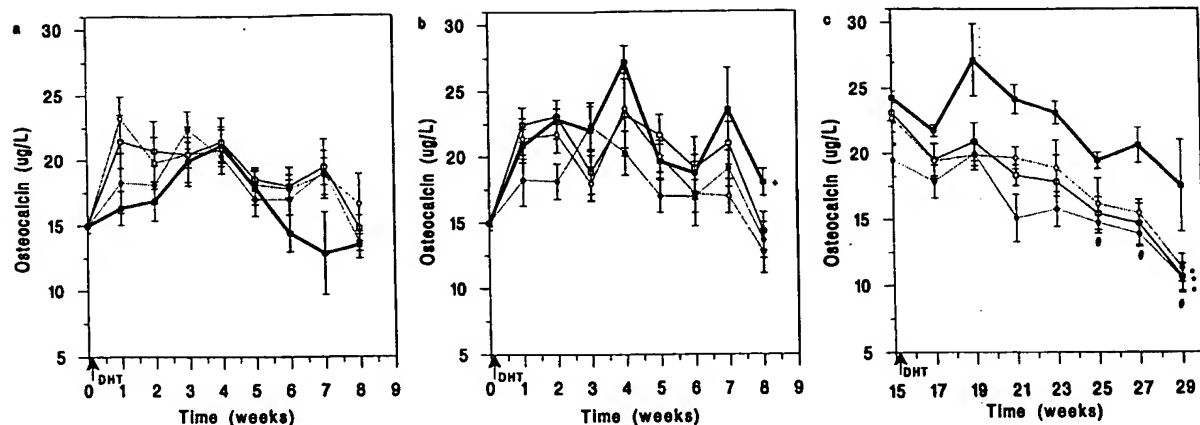


FIG. 2. Changes in serum osteocalcin ($\mu\text{g/L}$) in sham (a) and oophx (b) rats administered DHT from the time of operation and in oophx (c) rats administered DHT between 15 and 29 weeks following oophorectomy. (■) Vehicle, (□) 40 mg DHT, (◇) 80 mg DHT, (◆) 160 mg DHT. * $p < 0.001$ versus sham (a) within dosage group; * $p < 0.001$ versus vehicle within operation group; * $p < 0.005$ versus week 1 within operation group.

TABLE 2. EXPERIMENT 1: BIOCHEMICAL VARIABLES FOLLOWING DHT ADMINISTRATION FROM 0 TO 8 WEEKS POSTOPERATION

Variable	Operation	Vehicle	40 mg DHT	80 mg DHT	160 mg DHT
Hydroxyproline/creatinine ($\mu\text{mol}/\text{mmol}$)	sham	9.8 (0.4)	13.0 (0.8)	14.4 (1.1)	14.5 (1.3)
	oophx	12.5 (0.6) [†]	13.1 (0.5)	12.5 (0.8)	13.2 (0.5)
Deoxypyridinoline/creatinine (nmol/mmol)	sham	26.6 (2.9)	43.5 (5.3)	36.9 (9.0)	24.1 (7.6)
	oophx	33.7 (4.9) [*]	35.8 (3.9)	45.3 (8.6)	35.8 (5.0)
TmCa* (mmol/l GF)	sham	2.61 (0.07)	2.86 (0.07)	2.89 (0.07) [§]	2.97 (0.01) [§]
	oophx	2.72 (0.08)	2.88 (0.06)	3.16 (0.08) [§]	2.97 (0.07) [§]
Ionised calcium (mmol/l)	sham	1.34 (0.01)	1.31 (0.01)	1.31 (0.01) [§]	1.31 (0.01) [§]
	oophx	1.33 (0.01)	1.32 (0.01)	1.32 (0.01) [§]	1.31 (0.01) [§]
Serum creatinine (mmol/l)	sham	0.050 (0.002)	0.044 (0.002) [§]	0.046 (0.002) [§]	0.044 (0.002) [§]
	oophx	0.047 (0.003) [†]	0.042 (0.001) [§]	0.042 (0.001) [§]	0.038 (0.001) [§]

DHT dose is milligrams per kilogram of body weight. Values are mean (SE) of weeks 0–8. *TmCa, maximum tubular reabsorption of calcium; [†] $p < 0.001$ versus sham; [‡] $p < 0.05$ versus sham; [§] $p < 0.05$ versus vehicle within operation group; ^{||} $p < 0.05$ versus 160 mg DHT within operation group.

Serum creatinine decreased following oophorectomy ($p < 0.05$) and was decreased by DHT in both sham and oophx rats ($p < 0.05$) (Table 2). Body weight increased over time ($p < 0.005$); however, it was unaffected by DHT (data not presented). No relationship was observed between weight and serum creatinine (data not presented). Serum alanine aminotransferase (ALT) and albumin were unchanged throughout the duration of the experiment (mean ALT \pm SEM [U/l]; sham vehicle, 17.2 ± 4.7 ; sham DHT, 14.5 ± 2.6 ; oophx vehicle, 19.1 ± 4.9 ; oophx DHT, 20.2 ± 3.0 ; mean albumin SEM (g/l); sham vehicle, 34.6 ± 0.6 ; sham DHT, 33.9 ± 0.2 ; oophx vehicle, 33.3 ± 0.8 ; oophx DHT 33.8 ± 0.2).

Experiment 2

Serum alkaline phosphatase was increased as a result of DHT administration independent of dose ($p < 0.05$), and

this effect was time dependent with maximal serum levels not occurring until 14 weeks after commencing treatment (29 weeks postoperation) ($p < 0.001$) (Fig. 1c). Serum osteocalcin was decreased following DHT treatment ($p < 0.01$). This effect was also independent of dose but was time dependent with maximal suppression occurring between weeks 10 and 14 after commencing treatment (25 and 29 weeks postophorectomy; $p < 0.001$) (Fig. 2c).

Urine deoxypyridinoline/creatinine was decreased by DHT treatment ($p < 0.001$), although urine hydroxyproline/creatinine was unaffected (Table 3). The fall in urine calcium/creatinine following DHT treatment did not reach statistical significance ($p = 0.13$) (Fig. 3c). TmCa was elevated by DHT treatment ($p < 0.05$) (Table 3) and this effect was time dependent ($p < 0.025$) (data not presented), but again independent of dose. Serum ionized calcium (Table 3), alanine ALT, albumin, and body weight were unaffected by DHT administration and were unchanged

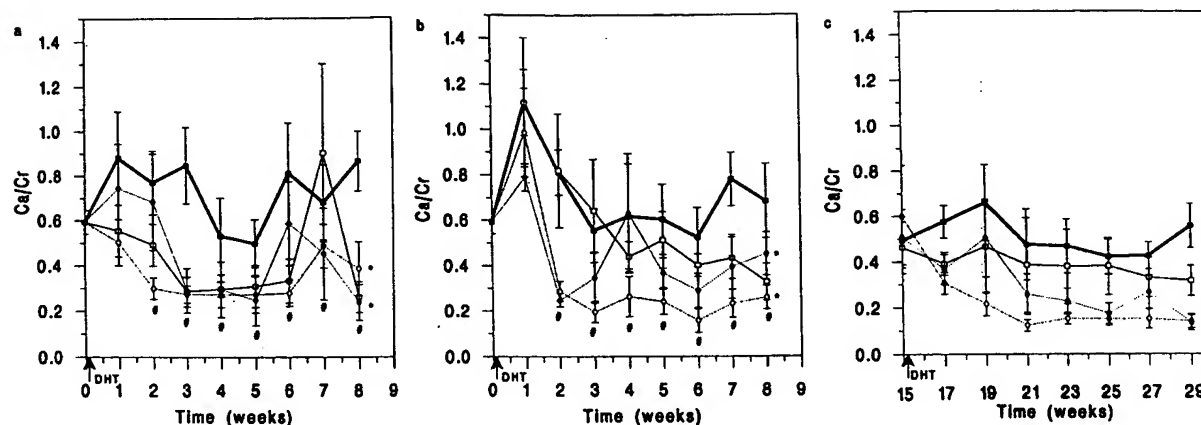


FIG. 3. Changes in urine calcium/creatinine (mmol/mmol) in sham (a) and oophx (b) rats administered DHT from the time of operation and in oophx (c) rats administered DHT between 15 and 29 weeks following oophorectomy. (■) Vehicle, (□) 40 mg DHT, (◇) 80 mg DHT, (◆) 160 mg DHT. * $p < 0.005$ versus vehicle within operation group; # $p < 0.005$ versus week 1 within operation group.

TABLE 3. EXPERIMENT 2: BIOCHEMICAL VARIABLES FOLLOWING DHT ADMINISTRATION FROM 15 TO 29 WEEKS POSTOOPHORECTOMY

Variable	Vehicle	40 mg DHT	80 mg DHT	160 mg DHT
Hydroxyproline/creatinine ($\mu\text{mol}/\text{mmol}$)	10.5 (0.3)	10.0 (0.3)	10.5 (0.3)	11.7 (0.5)
Deoxypyridinoline/creatinine (nmol/mmol)	46.7 (3.0)*	45.7 (5.4) [†]	37.3 (5.0) [†]	27.0 (1.9)
TmCa* (mmol/l GF)	2.68 (0.04)	3.0 (0.04)	3.17 (0.06)*	3.09 (0.05)*
Ionized calcium (mmol/l)	1.35 (0.01)	1.35 (0.01)	1.36 (0.01)	1.35 (0.01)
Serum creatinine (mmol/l)	0.042 (0.001)	0.036 (0.001)*	0.037 (0.001)*	0.037 (0.001)*

DHT dose is milligrams per kilogram of body weight. Values are mean (SE) weeks of 15–29. *TmCa, maximum tubular reabsorption of calcium; [†] $p < 0.05$ versus 160 mg DHT within operation group; * $p < 0.05$ versus vehicle.

throughout the experiment (mean ALT \pm SEM [U/l]; oophx vehicle, 15.7 ± 1.6 ; oophx DHT, 20.0 ± 1.5 ; mean albumin SEM (mmol/l); oophx vehicle, 33.8 ± 0.4 ; oophx DHT, 34.7 ± 0.2). Serum creatinine decreased upon DHT treatment ($p < 0.005$) (Table 3). No relationship was identified between serum creatinine and body weight (data not presented).

DISCUSSION

DHT treatment in osteopenic oophx rats results in a net gain in bone volume rather than the prevention of further bone loss.⁽⁵⁾ The accrual of bone is due to an increase in the bone formation rate, with increased surface extent of fluorochrome labels resulting in both increased trabecular thickness and number in the tibiae⁽⁵⁾ and stimulation of periosteal bone formation in the femur.⁽⁸⁾ At high doses of DHT, comparable to those used in the present study, the extent of osteoclast surface and number is reduced.⁽⁵⁾ It is of interest that at these high doses of DHT the bone formation rate does not differ from oophx control rats although, at least over the short term, bone mineral density

is significantly increased.^(5,8) In the present study, we have demonstrated significant biochemical changes with DHT treatment providing further information on the activities of androgens on bone and calcium metabolism.

An increase of serum alkaline phosphatase was observed in both sham and oophx rats following DHT administration either at operation or 15 weeks postoperation. The response was rapid, with stimulation evident after 2 weeks in the groups receiving DHT at operation but slower in the osteopenic oophx rats with 14 weeks of treatment required to detect stimulation. No changes were detected in serum albumin levels, a protein synthesized by the liver, or alanine aminotransferase, a liver enzyme, suggesting that the effect of DHT is on bone, rather than liver alkaline phosphatase. This effect is consistent with the induction of alkaline phosphatase by DHT in isolated osteoblast cells in vitro which was blocked by hydroxyflutamide, confirming this activity is mediated by the androgen receptor.⁽¹⁸⁾ The resultant levels of alkaline phosphatase in both sham and oophx rats were comparable, suggesting that these dosages provided maximal stimulation of osteoblasts independent of ovarian status.

In contrast, DHT did not affect serum osteocalcin levels

in sham-operated rats and decreased osteocalcin in oophx rats only when administered at 15 weeks following oophorectomy. Therefore, a differential effect of this androgen on osteoblast products has been observed. It is proposed that alkaline phosphatase and osteocalcin synthesis reflect different aspects of osteoblastic activity,⁽¹⁹⁾ and therefore this discordant effect of DHT on their serum levels may indicate that androgens act at a specific stage of osteoblast maturation. These data are consistent with a model that DHT stimulates osteoblasts at this matrix maturation stage of development when alkaline phosphatase is synthesized without stimulating osteocalcin synthesis.

The effects of DHT on biochemical markers of bone resorption were similar to those observed on serum osteocalcin levels. Urine deoxypyridinoline was significantly reduced in osteopenic rats when DHT was administered 15 weeks postophorectomy, while urine hydroxyproline excretion was unaffected. Since deoxypyridinoline is considered to be a more specific marker of bone resorption⁽²⁰⁾ it may better reflect such changes compared with hydroxyproline. Nandrolone decanoate, a synthetic androgen, decreases osteocalcin in young and old rats when administered immediately following operation,⁽²¹⁾ which was attributed to an overall antiresorptive effect.

The differences between the effects of DHT on the biochemical variables when DHT was administered either at the time of operation or 15 weeks postophorectomy possibly reflects an interaction between DHT and the rate of bone resorption at the time of treatment. In osteopenic rats, the bone turnover rate as measured by osteoclast and osteoblast surface is not significantly different from that of sham rats in the cancellous bone of the tibiae at 120 days postoperation.^(8,22) When DHT was administered at the time of operation the effects on bone resorption may have been masked due to the increased number and activity of bone cells associated with the elevated bone turnover rate following estrogen deficiency.⁽²²⁾ In contrast, the osteopenic rats had a lower number of active bone cells at the time of treatment and thus the effect of DHT to decrease bone resorption was detectable. Serum osteocalcin was also decreased by DHT treatment in the osteopenic oophx rats, further supporting this theory.

Urine hydroxyproline, urine deoxypyridinoline, alkaline phosphatase, and osteocalcin were increased as a result of oophorectomy compared with ovary-intact rats receiving vehicle alone consistent with increased bone turnover. It is interesting to note that DHT abolished the effect of oophorectomy on bone turnover because the resultant levels of all bone biochemical variables measured following DHT administration were not significantly different between sham and oophx rats. The major factors contributing to this effect were the greater increase of alkaline phosphatase and the slight but not significant increase in serum osteocalcin and deoxypyridinoline and hydroxyproline excretion in sham rats compared with oophx rats. These results provide further evidence for an interaction between DHT and estrogen which has previously been reported in the oophx rat model⁽⁸⁾ and requires additional investigation.

Urine calcium excretion was significantly decreased, with DHT administered immediately following operation in both

sham and oophx rats, and, although not reaching statistical significance in oophx rats when administered 15 weeks following oophorectomy, this trend was maintained. Nandrolone decanoate administration also decreases urine calcium excretion in postmenopausal women^(2,3) and in young oophx rats.⁽²¹⁾ The decrease in urine calcium observed in the present study was associated with an increase in the tubular reabsorption of calcium in the kidney, suggesting a direct action of DHT on the kidney in agreement with the findings of Need and colleagues in postmenopausal women.⁽⁴⁾ Ionized calcium was significantly decreased in sham and oophx rats administered DHT at the time of operation although no effect was observed when DHT was administered to the osteopenic oophx rats. This may reflect increased calcium incorporation into bone with increased bone formation. The ability of DHT to conserve calcium at the level of the kidney contributes to the availability of calcium for incorporation into bone and the subsequent increase in bone volume.

In summary, this study has demonstrated that even at high doses DHT exerts a stimulatory effect on alkaline phosphatase levels with a decrease in ionized calcium levels consistent with stimulation of bone formation. There was no increase in body weight or serum creatinine levels suggesting that this action was a direct effect on bone cell activity rather than by stimulation of muscle tissue with an indirect effect on bone. DHT also suppressed urine deoxypyridinoline excretion and serum osteocalcin levels in osteopenic oophx rats, indicating an antiresorptive action in female rats. This action appears to be relatively weak since it was not detectable when DHT was administered immediately following either sham or oophorectomy operations. In addition, DHT increased the renal tubular reabsorption of calcium, suggesting a direct effect on the kidney. Since DHT is not metabolized to estrogen, each of these actions must be mediated by the androgen receptor. However, the further understanding of these pleiotropic activities and the possible interactions between DHT and the rate of bone resorption and/or estrogen warrants further study, particularly with respect to the effects of DHT on bone cell gene expression.

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Andropause: Clinical Implications of the Decline in Serum Testosterone Levels With Aging in Men

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IT is now well accepted that serum testosterone (T) levels decline progressively with aging in men (1–5). This decline is associated with alterations in body composition; diminished energy, muscle strength, and physical function; reduced sexual function; depressed mood; and decreased cognitive function. Similar changes occur in young men with androgen deficiency and are improved with T replacement therapy. However, the physiological and clinical significance of the aging-associated decline in serum T levels in men is unclear, particularly because T levels may remain within the normal range for young men. From a practical clinical standpoint, it is most appropriate to define “andropause” as an age-related decline in serum T levels in older men to below the normal range in young men that is associated with a clinical syndrome (i.e., symptoms and signs) consistent with androgen deficiency. The decline in T levels is a consequence both of aging per se and age-associated comorbid illnesses and medications that are used to treat them (6–13). However, regardless of the etiology, androgen deficiency may contribute, at least in part, to age-related decrements in physiological function and may be associated with a clinical syndrome.

Andropause has also been referred to by some as “androgen deficiency in the aging male (ADAM),” “partial androgen deficiency in the aging male (PADAM),” or “aging-associated androgen deficiency (AAAD).” The term “male menopause” is inappropriate because there is no interruption or cessation of menses, and “viropause” is inaccurate because there is no loss of virilization. “Male climacteric” refers to the syndrome of endocrine, somatic, and psychic changes that occur in normal men with aging. This term emphasizes the multidimensional nature of age-related changes, including decreases in other hormones such as growth hormone (GH), insulin-like growth factor-1 (IGF-1), dehydroepiandrosterone (DHEA), and melatonin (1,14–17), but it does not relate aspects of the male aging syndrome specifically with androgen levels. The term “andropause” is not completely accurate because androgen secretion does not cease altogether. However, because it is the only term that relates the syndrome of age-related physiological changes with the gradual and progressive decline in T levels that occurs with aging, andropause will be used in this review. Andropause is a term also used commonly by experts in the field and by lay persons because it retains some analogy to menopause in women.

PHYSIOLOGICAL BASIS OF ANDROPAUSE

Age-Related Decline in Serum T Levels

In both cross-sectional (5,6,18–38) and longitudinal studies (39–42), beginning in the third decade, aging is associated with a gradual and progressive decline in serum T levels at a rate of approximately 1% per year. As a result, ~20% of men older than 60 and ~50% of men older than 80 years of age have serum total T levels below the normal range for young men (39,43,44).

Circulating T is approximately 98% bound to serum proteins, predominantly to sex hormone-binding globulin (SHBG), the major binding protein for T in blood, and albumin (45–47). Only 1% to 2% of T in circulation is completely unbound or free. Because T is bound with high affinity (i.e., tightly) to SHBG, SHBG-bound T is not available to most tissues for action. In contrast, T is bound with low affinity (weakly) to albumin, so both albumin-bound and free T are bioavailable to most tissues for action. Because the concentration of SHBG increases with aging, serum-free T and bioavailable T (free plus albumin-bound T) concentrations decline more markedly than total T levels with aging (13, 16,20,24,26,27,29,31,33,36,39,41,48). Therefore, a much larger percentage of older men have levels of these biologically active fractions of circulating T below the normal range for young men (39,49).

Age-related and other alterations in SHBG have important practical clinical implications in the diagnosis of androgen deficiency. Because total T assays measure both free T and T bound to SHBG and albumin, alterations in SHBG and/or albumin result in changes in total T levels in the same direction. Measurements of bioavailable or free T are not affected by alterations in SHBG. Therefore, they provide a better assessment of biologically active T in blood, especially in common clinical states such as aging in which SHBG increases or moderate obesity in which SHBG decreases (47,50–52).

Because SHBG levels increase with aging, many older men with low-normal total T levels have free or bioavailable T levels that are below the normal range for young men. Therefore, measurements of bioavailable or free T using ammonium sulfate precipitation or equilibrium dialysis, respectively, or calculated from measurements of total T and SHBG are recommended to diagnose androgen deficiency in older men. Unfortunately, these measurements of

free and bioavailable T are not usually performed in local laboratories and are only available through commercial reference laboratories. Most local laboratories measure free T using a solid-phase direct analog immunoassay kit. Although free T measurements using this method correlate with those using equilibrium dialysis, values obtained differ substantially [e.g., by more than an order of magnitude in women (53)] from those obtained by equilibrium dialysis or calculated from SHBG, and vary directly with alterations in SHBG levels (52–56). Therefore, free T measurements using direct analog immunoassay kits may not provide useful clinical information beyond that of total T levels. They tend to underdiagnose older men with androgen deficiency and overdiagnose androgen deficiency in men with low SHBG levels (e.g., moderately obese men). Free T levels measured using a direct analog immunoassay should not be used in situations where SHBG levels may be altered (e.g., older men).

Decline in Both Testis Function and Hypothalamic GnRH Regulation With Aging

The decline in serum T levels with aging is due both to impaired testis production of T and hypothalamic secretion of gonadotropin-releasing hormone (GnRH) resulting in inadequate stimulation of luteinizing hormone (LH) secretion by the pituitary gland.

Older men demonstrate a decrease in the number of Leydig cells (57–59), the cells of the testis that produce T, reduction in basal T production (60,61), and marked decreased in T secretion by the testis in response to maximal stimulation by administration of human chorionic gonadotropin (hCG), an LH-like hormone (62–66). The impact of reduced T production on circulating T levels is lessened by the decrease in metabolic clearance of T that also occurs with aging (35). The normal circadian variation in serum T levels with peak concentrations in the morning is blunted in healthy older men compared to young men (65,67–72), suggesting an alteration in the hypothalamic circadian pacemaker function. Because of age-related blunting of the normal circadian variation in T levels, early morning serum T levels are lower but late afternoon values are more similar in older compared with young men (23).

Function of the seminiferous tubule compartment of the testis also declines with aging. In older compared with young men, spermatogenesis assessed histologically is reduced (58,73,74), but ejaculated sperm concentration is unchanged or increased as a result of diminished ejaculatory volume and frequency (28,75). The number of sperm with normal motility and morphology also decreases but in vitro fertilizing capacity is relatively well preserved in older men (28,76). Despite overall well-preserved fertility potential (77) and documented instances of paternity in men older than age 90 years, overall fertility rates decline with age (78,79), largely as a result of diminished sexual activity (80). With older paternal age, the risk of inherited autosomal dominant diseases increases in offspring (79,81). The number of Sertoli cells (57,82), seminiferous tubule cells that support spermatogenesis, and serum levels of inhibin B, a Sertoli cell peptide product responsible for feedback inhibition of follicle-stimulating hormone (FSH) secretion from

the pituitary gland, decrease with aging (83,84). Most of the decline of inhibin B levels appears to occur by middle age with stable concentrations from middle to old age.

In both cross-sectional and longitudinal studies, the decline in serum T levels with aging is associated with a gradual increase in serum gonadotropins, FSH, and to a lesser extent, LH concentrations (19,25,41,85–87). Although gonadotropin levels increase with aging, they often remain within the wide normal range for younger men. The resulting hormonal pattern of a low serum T and normal gonadotropin levels suggests concomitant hypothalamic-pituitary dysfunction in conjunction with primary testicular failure in aging men. Low serum T and normal gonadotropin levels, consistent with secondary hypogonadism, are found commonly during the work-up of older men with symptoms of androgen deficiency (see below) (88).

Detailed studies of pulsatile gonadotropin secretion provide indirect evidence for age-related alterations in pulsatile GnRH secretion from the hypothalamus. Compared with normal men, young hypogonadal men with low serum T levels demonstrate an increase in LH pulse frequency and amplitude associated with diminished T negative feedback (89,90). Compared with young men, healthy older men with low serum T levels demonstrate an abnormal LH pulse frequency, reduced LH pulse amplitude, and more disorderly LH secretion, suggesting an age-associated impairment of the hypothalamic GnRH pulse generator (71,91–98). Basal FSH secretion and pulse amplitude increase, but orderly secretion of FSH is maintained in older compared with young men (99–101). Older men also demonstrate an attenuated stimulation of gonadotropin secretion induced by naltrexone or naloxone, opioid receptor antagonists, suggesting altered central nervous system (CNS) endogenous opiate regulation of GnRH secretion with aging (97,102). The sensitivity of gonadotropin suppression to T negative feedback is increased (103–105), and gonadotropin responsiveness to androgen deprivation induced by an androgen receptor antagonist (flutamide) or androgen synthesis inhibitor (ketoconazole) is attenuated in older compared with young men (106,107).

Compared with young men, older men demonstrate slightly diminished gonadotropin responsiveness to acute GnRH (85,87,108–110) but a normal LH response to chronic pulsatile GnRH administration (111), suggesting that pituitary gonadotropin secretion remains intact with aging. Together, these findings suggest that aging is associated with impairments in both testis function and hypothalamic GnRH regulation of gonadotropin secretion.

Age-Related Alterations in Androgen Action and Active Metabolism of T

Besides the limited studies of T negative feedback mentioned previously, a systematic evaluation of age-related changes in androgen action in androgen-responsive target organs has not been performed. Androgen receptor gene expression in the CA1 region of the hippocampus and the number of androgen receptor binding sites in genital skin are decreased in older compared with young men (112–114). Androgen receptor expression and nuclear androgen receptor levels in the prostate are unchanged in older men with-

out benign prostatic hyperplasia (BPH) and are similar to those in young men (115–117). However, prostate androgen receptor expression is reduced, and nuclear androgen receptor levels are increased in older men with BPH compared with young men.

The length of trinucleotide CAG repeats in the androgen receptor gene is variable and is associated with differences in transcriptional activity, with a shorter CAG repeat length associated with greater androgen receptor activity and possibly overall greater androgen action (118). In the Massachusetts Male Aging Study (MMAS), serum total and free T levels were found to be associated with the CAG repeat length in the androgen receptor gene (40). Older men with lower serum T levels had an androgen receptor genotype characterized by a shorter CAG repeat length, suggesting overall greater androgen activity. It is hypothesized that, in older men with shorter CAG repeat length, increased androgen action at the level of the hypothalamic-pituitary axis may result in greater feedback suppression of gonadotropin and, in turn, endogenous T secretion. This may be an intrinsic mechanism that underlies the physiological decline in serum T levels with aging. A shorter CAG repeat length in the androgen receptor gene also has been associated with an increased risk and severity of BPH and prostate cancer (119–125) and an earlier age at diagnosis and aggressiveness of prostate carcinoma (126–129).

Androgen action is not simply a function of androgen receptor expression in target tissues and CAG repeat length, but involves a complex interaction among androgen ligands such as T, the androgen receptor, and tissue-specific coactivators and corepressors with androgen-response elements in specific genes (130,131). Age-related alterations of the latter and other transcription factors in androgen target tissues and their effects on androgen action have not been investigated. However, the preliminary findings reviewed suggest that, in addition to circulating T levels, age-associated changes in androgen action may play important roles in the alterations of physiological function that occur with normal aging and in the pathophysiology of age-related pathologies.

T is actively metabolized to the potent estrogen, estradiol (E2), by the enzyme aromatase, which is located primarily in adipose tissue, and to 5 alpha-dihydrotestosterone (DHT), a more potent androgen than T, by the enzymes 5 alpha-reductase type 1 and 2, which are located predominantly in skin and the prostate (132–134). Many of the actions of T are mediated, at least in part, by its active metabolites, E2 (e.g., bone, brain, and lipids) and DHT (e.g., prostate). Despite declining T levels, serum total E2 and DHT levels do not change or decrease only slightly with aging (24,26,34,37,38,135–139). This suggests that, with aging, there is a relative increase in aromatization of T to E2 (perhaps due to increased adipose tissue mass) and 5 alpha-reduction of T to DHT and/or reductions in the metabolic clearance of E2 and DHT. Because serum SHBG levels increase with aging, serum bioavailable or free E2 and DHT levels would be expected to decrease with aging. The physiological significance of bioavailable E2 and DHT is not clear. However, recent studies suggest that bioavailable E2 levels decline with aging and correlate better than T with bone mineral density in men (26,137,139,140).

Serum markers of peripheral androgen action such as 3 alpha-, 17 beta-androstenediol glucuronide (3 alpha-diol G) decrease markedly with aging, suggesting an overall decline in the total circulating androgen pool (24,138,141). Tissue concentrations of DHT decrease within the epithelial compartment and E2 increase within the stromal compartment of the normal and BPH prostate gland with aging, emphasizing the importance of active metabolism of T in androgen target organs and within specific regions of these organs (142–144).

Age-Related Comorbid Illnesses and Medications Suppress Serum T Levels Further

In addition to the decline in serum T levels associated with healthy aging, age-related comorbid illnesses (e.g., chronic renal, liver, or pulmonary disease, malignancy) increase, and the use of certain medications that are often used to treat these illnesses (e.g., glucocorticoids and CNS-active medications) and malnutrition that is often associated with illness suppress serum T levels even further (11,12,51,145–148). Compared with community-dwelling healthy older men, old men with significant illnesses, such as cancer or stroke, and those in an inpatient rehabilitation unit or nursing home have substantially lower serum T levels (6–10,21). These sicker old men also have a higher prevalence of T levels below the normal range for either healthy young (~60–90%) or older men (~20–30%).

Age-Related Decline in Adrenal Androgens

Serum concentrations of DHEA, a weak adrenal androgen that is a precursor of T, decline more rapidly and more profoundly than those of T with aging (149–152). This has been referred to as “adrenopause.” However, the physiological significance of circulating DHEA is unclear at this time. Preliminary controlled studies of DHEA treatment failed to demonstrate significant clinical effects in older men and conflicting effects on general well being (149,153–157). Therefore, the term adrenopause is reserved for the age-related decline in T, the major circulating androgen in blood.

Age-Associated Physiological Changes Consistent With Androgen Deficiency

Aging is associated with a number of changes in physiological functions, many of which are regulated by androgens. Physiological alterations that are associated with the age-related decline in serum T levels include decreased lean body mass and muscle mass (predominantly in fast twitch type II muscle fibers) (158–174); reduced muscle strength and power (164,175–180); decreased physical function, aerobic capacity, and balance (175,181–187); increased risk of falls and loss of independent living; increased fat mass during middle to old age, in particular increased amounts of visceral adiposity, which is associated with insulin resistance and increased risk of type 2 diabetes mellitus, hypertension, and atherosclerotic vascular disease, followed by stable or decreased fat mass in very old age (159,188–193); decreased bone mineral density (BMD) and increased risk of osteoporosis and fractures (194–201); decreased skin thickness and body hair, and poor wound healing (202,203);

diminished vigor, energy, and general well being; irritability and depressed mood (204); decreased sexual function (reduced libido, sexual activity, and erectile function) (80,205–208); impaired concentration and cognitive function (209,210); sleep disturbances and impaired sleep quality (211,212); and decreased hematopoiesis (213,214).

Similar alterations in physiological function occur in younger hypogonadal men with androgen deficiency and T replacement therapy: increases lean body mass, muscle mass, and strength; decreases body fat mass; improves energy, well being, mood, and libido; increases spontaneous erections during sleep (nocturnal penile tumescence) and induced by sexual thoughts; improves sexual function; and increases erythropoiesis and hematocrit (215–268). Therefore, it is hypothesized that the decline in serum T levels with aging contributes, at least in part, to these age-related alterations in physiological function, especially in older men with serum T levels below the normal range for young men. Although not uniform, most descriptive studies find a correlation between serum T levels and most of these physiological functions, independent of age.

Some descriptive studies find a positive correlation between T levels and lean body mass, and muscle mass and strength in older men (7,139,269), while others do not (269–272), and most studies (273–277) find an inverse correlation between T and total or abdominal fat mass, suggesting a relationship between serum T and age-related alterations in body composition and muscle strength. Furthermore, serum T levels are lower in men with type 2 diabetes mellitus, and low T levels are associated with a higher risk of developing type 2 diabetes (278–282). However, many studies investigating the association of T levels with total or visceral adiposity and diabetes may have been confounded by the use of total or free T assays that were affected by SHBG concentrations, which are significantly lower in moderately obese men (283,284).

Most epidemiological studies find a positive correlation between free T levels within the physiological range and high-density lipoprotein (HDL) cholesterol levels, and an inverse correlation between T concentrations and hypertension, insulin and glucose levels, prothrombotic factors, atherosclerotic vascular disease, and the presence or severity of coronary artery disease (CAD) (31,285–303). In prospective studies, no correlation is found between T levels and CAD disease incidence (285). No studies have reported an association between T and cardiovascular mortality. Therefore, epidemiological studies suggest a protective or neutral rather than an adverse effect of T on heart disease risk.

A relatively weak positive correlation is found between free, bioavailable, or total T levels and BMD and fracture risk in some studies (137,139,273,304–310), but no correlation is found in others (140,311–319). In recent studies, a stronger correlation is found between bioavailable E2 levels and BMD and fracture risk than between T and these outcomes, suggesting that the age-related reduction in bioavailable E2 levels may be a more important determinant of bone loss with aging in men than T levels (139,140,306,308,311,318,320,321). The findings of severe osteoporosis in men with estrogen resistance or deficiency caused by estrogen receptor or aromatase gene mutations, respectively, and an

increase in BMD with E2 treatment in a man with aromatase deficiency provide strong support for a vital importance of E2 in developing and maintaining normal bone mass (322–328). In older men, administration of an aromatase inhibitor increases markers of bone resorption, and E2 replacement in these men decreases markers of bone resorption, suggesting an important role for T to E2 conversion in the prevention of bone resorption (329,330). However, men with androgen resistance caused by androgen receptor gene mutations have reduced BMD, suggesting that androgens also play an important role in the development and maintenance of bone mineral content in men (331–333). The specific roles of T and E2 in regulating bone turnover were investigated recently in older men with T and E2 deficiency (induced by a GnRH agonist plus an aromatase inhibitor) treated with either physiological T or E2 replacement (334). E2 but not T was found to be dominant in preventing bone resorption, whereas both T and E2 were found to be important in maintaining bone formation. Because E2 is derived from aromatization of T, it is likely that the age-related decline in serum E2 levels is related, at least in part, to the reduction in its substrate, T, with aging. Finally, low T levels are a risk factor for hip fractures in older men (335–337).

Most descriptive studies also find significant associations between T levels and aspects of brain function. In a recent study, an inverse correlation was found between bioavailable T and depression score, suggesting a role for T in the regulation of mood (338). The correlation between T levels and libido and sexual activity is weak (339–345), and such an association is not found in some studies (346). This is consistent with the findings that relatively low serum T levels are required to sustain sexual interest and desire (219,347,348). Although androgen deficiency may contribute to sexual dysfunction, it is rarely the only or major cause of erectile dysfunction in older men. Erectile dysfunction is most commonly due to vascular disease, neuropathy, and medications (88,349,350). T levels are associated with sleep efficiency and architecture (351) and inversely with measures of psychosocial stress (352). Finally, a correlation between bioavailable T and general or spatial cognitive function has been reported, suggesting a potentially very important role for T in the maintenance of cognition and memory (353–357).

Given the multifactorial nature of age-related physiological alterations (see below), it is not surprising that there is a relatively weak correlation between serum T levels and these physiological changes that occur with aging. In fact, recent large epidemiological studies suggest that much of the age-associated decline in serum T levels is attributable to age-related comorbid illnesses, medications, and lifestyle (8,13,358–360).

Multifactorial Etiology of Age-Related Physiological Changes

It would be naïve to assume that age-associated androgen deficiency is the only cause of the physiological changes that occur with aging. As is the case for many geriatric syndromes, the etiology of most age-related alterations in physiological function is multifactorial, and many of the factors

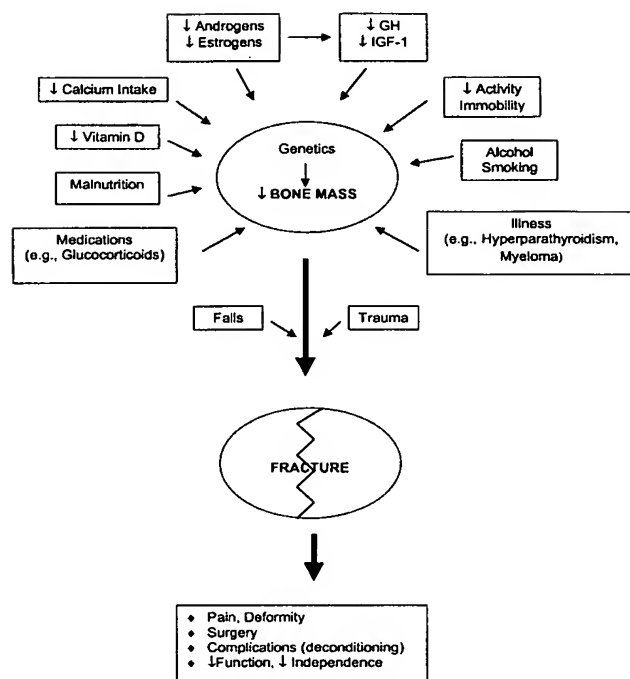


Figure 1. Schematic diagram of the multiple factors that may contribute to decreased bone mass and abnormal bone architecture in older men. These include low free or bioavailable T and E2 levels, low growth hormone (GH) and insulin-like growth factor-1 (IGF-1) concentrations, reduced calcium intake, vitamin D deficiency, malnutrition, use of medications that reduce bone mass (e.g., glucocorticoids, or anticonvulsants), decreased exercise or activity, immobility (e.g., from severe arthritis), lifestyle (excessive alcohol use or tobacco smoking), illnesses that reduce bone mass (e.g., primary or secondary hyperparathyroidism or multiple myeloma), and genetic factors that influence bone metabolism. Reduced bone mass and abnormal bone architecture predispose to fracture following falls or trauma, and fractures may cause significant clinical morbidity and mortality [e.g., pain, deformity, need for surgery, complications (e.g., deconditioning or pneumonia), and loss of function and independence].

that contribute to physiological decline are modifiable or treatable. Therefore, optimal clinical management of these changes requires careful attention to all potential etiological factors. For example (Figure 1), in addition to low T levels, the age-related decrease in BMD and abnormalities in bone architecture may be due to low E2 concentrations; low GH and IGF-1 levels; inadequate calcium intake; vitamin D deficiency; poor nutrition; use of medications (e.g., glucocorticoids or anticonvulsants); lack of exercise or inactivity due to weakness or immobilization (e.g., as a result of severe osteoarthritis); lifestyle (excessive alcohol intake, smoking); age-related illness (e.g., primary and secondary hyperparathyroidism, multiple myeloma); predisposing genetic background; and/or low peak bone mass during puberty and young adulthood as a result of prolonged illness, medications, or delayed puberty (361). The latter two potential etiologies emphasize the need for geriatricians and gerontologists to consider aging as starting from conception and

extending throughout the life span of an individual, not just from young adulthood to old age. Finally, it is important to recognize the essential contribution of falls and trauma to the major clinical consequence of decreased bone mass and abnormal bone architecture, fracture, which may lead to pain, deformity, need for surgery, and associated complications (e.g., deconditioning or pneumonia), and loss of function and independent living.

Interactions among the potential etiological factors that contribute to age-related physiological decline increase the clinical complexity and highlight the importance of using a multifactorial approach in managing older patients. For example, low serum T levels may contribute to reductions in E2, GH, and IGF-1 levels (362), decreased exercise or inactivity as a result of weakness and/or poor motivation, and increased risk of falls related to muscle weakness and poor balance. T treatment may increase BMD indirectly by increasing E2, GH, and IGF-1 levels and physical activity, and reducing the likelihood of falls by improving muscle strength, balance, and spatial cognition. Conversely, many of the factors that decrease BMD (e.g., poor nutrition, medications, comorbid illnesses, and excessive alcohol intake) also may contribute to the reduction in serum T levels. Correction of poor nutrition, discontinuation of certain medications such as glucocorticoids, treatment of comorbid illnesses, and discontinuation of alcohol abuse may increase serum T levels and obviate the need for T treatment. In the clinical approach to older men with low serum T levels, a similar multifactorial evaluation of possible etiological factors contributing to other age-related physiological alterations should be adopted.

Potential Consequences and Importance of Age-Associated Physiological Changes

Age-associated changes in physiological function have important potential consequences, including reduced physical function, endurance, and activity; increased risk of disease (e.g. coronary heart disease, diabetes mellitus, osteoporosis); diminished quality of life; impaired balance and increased risk of falls; impaired ability to regain function after an acute illness; and, most importantly, increased susceptibility to frailty (2,363). In turn, frailty may lead to a loss of independence, chronic disability, and a need for assisted living or long-term care; severe psychological and socioeconomic sequelae; and an increase in mortality.

A major clinical focus of geriatric medicine is the prevention of frailty. A multifaceted approach is employed to prevent frailty in elderly persons. This includes interventions to prevent acute and chronic diseases (e.g., influenza vaccination, smoking cessation); prevent falls and injury (e.g., discontinuation of medications that predispose to falls and hip protectors); adequately treat acute and chronic diseases; identify and treat conditions that impede recovery of function (e.g., delirium and depression); improve physical conditioning (e.g., aerobic and resistance exercise); improve nutritional intake; and replace hormonal loss (e.g., estrogen replacement therapy in postmenopausal women). With regard to the more gradual and less profound age-related decline in serum T levels, major unanswered clinical questions are whether T replacement therapy of older men will im-

prove functional status, prevent disease, increase the quality of life, and, most importantly, reduce the risk of frailty (2,364).

POTENTIAL BENEFITS AND RISKS OF T REPLACEMENT THERAPY IN OLDER MEN

When considering T replacement therapy in older men, the potential benefits of T treatment must be weighed against the possible risks (365). The potential benefits and risks of T replacement therapy in older men (Table 1) are based mostly on the clinical studies of the effects of T replacement in severely androgen-deficient, young, hypogonadal men (discussed in the previous section) and a small number of controlled trials of T treatment in older men (discussed in the following section).

Potential benefits of T replacement therapy in older men include increased lean body mass; decreased fat mass and visceral adiposity, and reduced risk of diabetes mellitus; increased muscle mass and strength; increased BMD and reduced risk of osteoporosis and fractures; increased body hair and skin thickness, and improved wound healing; improved physical function, aerobic capacity, and balance; improved libido and sexual function; improved feeling of well being and improved energy; reduced irritability and depressed mood; improved concentration and cognitive function; improved sleep quality; increased hematopoiesis and hematocrit; and decreased risk of CAD.

Short-term risks of T administration in older men include stimulation of erythropoiesis, resulting in excessive erythrocytosis and, if severe, increased blood viscosity and risk of thrombotic complications (e.g., stroke and myocardial infarction); induction or worsening of obstructive sleep apnea; worsening of peripheral edema; and development of gynecomastia.

The most concerning long-term potential risk of T administration in older men is the stimulation of previously unrecognized local or metastatic prostate cancer (366). Prostate

cancer is the most common malignancy in men, and many older men harbor microscopic foci of prostate cancer that do not become clinically apparent during their lifetime (367). In most (368–370) but not all (371) epidemiological studies, serum T levels were not associated with a risk of prostate cancer. However, because prostate cancer growth is initially androgen-dependent, there is concern that T treatment of older men will stimulate growth of preexisting subclinical (microscopic) prostate cancer to become clinically detectable. This concern is heightened by reports that find a high prevalence of prostate cancer (~25%) on surveillance biopsies of older men with low serum T, and normal prostate-specific antigen (PSA) levels and digital rectal examinations (372,373). However, biopsies were not performed in an age-matched control group of men with normal T levels in these studies, and in other studies, the prevalence of prostate cancer in older men with normal PSA levels and digital rectal examinations is comparable to those found in these reports (374). As a consequence of more intensive monitoring of digital rectal examinations and PSA levels, another underappreciated potential risk of T treatment in older men is the increased likelihood of detecting subclinical localized prostate cancer for which treatment is unclear. Even if subclinical disease discovered on biopsy does not affect overall prognosis, the potential medical, surgical, psychological, socioeconomic, legal, and ethical consequences of a diagnosis of subclinical prostate disease may be quite great.

Benign prostate growth is also androgen-dependent (375,376). At the time of puberty, prostate size increases in response to the increases in endogenous T production. Patients with severe prepubertal androgen deficiency have small, underdeveloped prostate glands, and if left untreated, these men do not develop BPH. Long-term T treatment of young hypogonadal men increases prostate size to volumes that are comparable to age-matched eugonadal men (377,378). In men with BPH, treatment with a 5 alpha-reductase inhibitor (e.g., finasteride, which inhibits the conversion of T to the potent androgen DHT) decreases prostate size, improves urine flow, reduces symptoms of BPH, and decreases the need for BPH-related surgery (379). Therefore, another potential long-term risk and concern of T treatment in older men is the stimulation of BPH growth and worsening of symptoms, urine flow, and urinary retention that may require invasive intervention, such as a transurethral resection of the prostate (TURP).

Although rarely an issue, T replacement may suppress already compromised sperm production in older men and potentially reduce fertility. Men have a higher risk of CAD than women. Also, treatment of severely androgen-deficient, young, hypogonadal men with physiological T dosages and eugonadal men with supraphysiological T dosages decreases HDL cholesterol levels, producing a more atherogenic profile (286,380–385). Therefore, there is concern by some that T replacement in older men may increase the risk of cardiovascular disease. As discussed previously, most epidemiological studies suggest that low T levels are associated with an increase in the prevalence and severity of CAD in men. However, in the absence of long-term studies to evaluate the effects of T on major cardiovascular outcomes such as coronary death, myocardial infarction, and stroke,

Table 1. Testosterone Treatment in Older Men: Potential Benefits and Risks

Potential Benefits	Potential Risks
Improve body composition	Erythrocytosis, hyperviscosity
↑ Lean body mass	Induce or worsen sleep apnea
↓ Fat mass, ↓ visceral fat	Worsen edema
↑ Muscle mass and strength	Gynecomastia
↑ BMD, ↓ fractures	Clinical prostate disease
↑ Hair, skin thickness	Worsen BPH requiring intervention
↑ Physical function	Hasten clinical prostate cancer
Improve brain function	Suppress sperm production
↑ Libido, sexual function	Increase cardiovascular risk
↑ Well being, energy	
↓ Irritability, depression	
↑ Cognitive function	
↑ Sleep quality	
Increase hematocrit	
Decrease cardiovascular risk	

Notes: ↑ Signifies a potential increase or improvement in the endpoint with testosterone treatment. ↓ Signifies a potential decrease or reduction in the endpoint with testosterone treatment. BMD = bone mineral density; BPH = benign prostatic hyperplasia.

the possibility remains that T therapy in older men may increase cardiovascular disease risk.

CLINICAL TRIALS OF T THERAPY IN OLDER MEN

Relatively few randomized controlled studies have been reported that investigate the effects of T treatment in older men (386–423). In these studies, a variety of T formulations and dosages were used to treat a relatively small number of mostly healthy older men, and differing methods were used to assess outcomes. However, these preliminary controlled studies suggest that T treatment in older men has beneficial effects on body composition (increase in lean body mass and decrease in fat mass), bone mineral density, LDL cholesterol, angina and exercise-induced cardiac ischemia, and possibly on muscle strength, sexual function, general well being, and aspects of cognitive function (Table 2).

No formal evaluation of the dose-response effects of T treatment on relevant outcomes has been performed in older compared to young, androgen-deficient men. Therefore, the impression that the effects of T treatment are attenuated in older men relative to young men is not supported by evidence. Also, most studies have been performed on relatively healthy older men with T levels in the lower part of the normal range or slightly below normal. Therefore, it is difficult to compare the effects of T therapy in these studies of

mildly androgen-deficient older men to those found in studies of more severely T-deficient young men.

Beneficial Effects of T Therapy in Studies of Older Men

In most controlled trials of older men, T treatment resulted in improvements in body composition. In both controlled (388,390,401,405,406,415,417,418,424) and uncontrolled (425) studies, the most consistent effects of T therapy in older men were an increase in lean body mass and/or a decrease in total fat mass. One study found a decrease in visceral fat mass and improvement in insulin sensitivity with T treatment (406). The effects of T supplementation in older men on muscle strength were more variable. Some studies found an increase in upper extremity grip strength (408,415,424), and one uncontrolled study found an increase on lower extremity strength with T therapy (425). However, other carefully performed controlled studies found no increase in either upper or lower extremity strength with T treatment of older men (388,391,394,405, 417,418). The lack of consistent effects of T therapy on muscle strength may be due to differences in the methods used to assess strength and the dosages and duration of T that were administered. In a preliminary report, timed walking and stair climbing improved in older men with short-term T administration (391). Self-assessment of physical function was maintained in older men treated with transdermal T compared to placebo for 3 years (417). Supraphysiological but not physiological T administration also has been reported to increase the secretion of another anabolic hormone, GH, in older but not young men (398). No controlled studies have investigated systematically the effect of T therapy in older men on muscle power, balance, or endurance, which together with strength play important roles in the maintenance of physical function in older men.

Controlled studies in which T was administered for at least 1 year demonstrated an increase in lumbar spine and hip BMD and prevention of bone loss in the femoral neck with T compared to placebo treatment in older men with low T levels (388,405,424). In one study, T prevented loss of BMD in the femoral neck despite calcium and vitamin D supplementation in all subjects (405). In another study that included older men with normal serum T levels and in which some but not all subjects received calcium and vitamin D (if dietary intake was inadequate), both T- and placebo-treated men exhibited an increase in BMD without a significant difference in response between the two groups (416). However, further analysis revealed that T treatment increased BMD in older men who had low serum T levels (<300 ng/dl) at baseline, suggesting that a beneficial effect of T treatment on BMD may occur only in older men with androgen deficiency. These studies emphasize the importance of considering other etiologies besides low T levels that may contribute to clinical manifestations consistent with androgen deficiency in older men (e.g., low calcium intake and vitamin D deficiency in older men with low BMD) (426). No controlled studies have evaluated the effect of T on bone architecture or the risk of falls that together with BMD contribute to the age-related increase in the risk of fractures in men. Furthermore, no studies have investigated sufficient numbers of older men for long

Table 2. Testosterone Treatment of Older Men: Summary of Controlled Studies

Benefits	Risks
↑ Lean body mass	↑ Hematocrit, risk of erythrocytosis
↓ Fat mass	η Sleep apnea
↑/η Grip and leg strength	η Clinical prostate disease
↑ BMD (if low T initially)	η BPH symptoms, size, urine flow
? ↓ Fractures	↑/η PSA (<4 ng/ml)
↑/η Sexual function, well being	? ↑ BPH intervention
↑ Libido	? ↑ Clinical prostate cancer
η Erectile dysfunction	η HDL cholesterol
↑ Well being, ? ↓ depression	? ↑ Cardiovascular events
↑/η Cognitive function	
↑ Spatial, working memory	
↑ Verbal memory	
η Visual memory	
? Slow dementia onset	
↓ Total and LDL cholesterol	
↓ Angina and exercise ischemia	
? ↓ Cardiovascular events	
? ↑ Physical function	
? ↓ Frailty	
? ↑ Quality of life	

Notes: Composite of benefits and risks of testosterone treatment in older men derived from references (385–422). ↑ Signifies that most controlled studies found an increase in the endpoint with testosterone treatment. ↓ Signifies that most controlled studies found a decrease in the endpoint with testosterone treatment. η Signifies that most controlled studies found no change in the endpoint with testosterone treatment. ↑/η Signifies that some controlled studies found an increase and others found no change in the endpoint with testosterone treatment. ? Signifies that controlled studies have not been performed to address the endpoint sufficiently. BMD = bone mineral density; LDL = low-density lipoprotein; BPH = benign prostatic hyperplasia; PSA = prostate-specific antigen; HDL = high-density lipoprotein.

enough (i.e., had sufficient statistical power) to determine whether T treatment decreases the incidence of fractures in older men.

T treatment in older men increased libido and sexual activity and improved energy and well being in some controlled studies (400,406,410,413,418,419) but not in others (388,389,403,404,408,415,417). An uncontrolled trial also reported improvements in energy, mood, well being, libido, and sexual activity (427). One controlled study found no effect of T therapy on clinical depression in older men (415). Other studies have not investigated the effect of T on depression in older men. In a recent small, double-blind, randomized, placebo-controlled study of depressed hypogonadal middle-aged men, short-term T administration (6 weeks) improved sexual function but did not improve depression scores compared to placebo (266). In preliminary placebo-controlled studies, certain domains of cognitive function, specifically spatial, verbal, and working memory, improved with T treatment in older men (392,403,404) but had no effect on general memory, recall, or verbal fluency (415). In one preliminary study, very short-term T administration (5 days) prevented the improvement in verbal fluency observed in placebo-treated older men (423). Studies have not investigated whether T treatment will slow the onset of clinical dementia in older men.

Most controlled studies of T therapy in older men found a decrease in total and low-density lipoprotein (LDL) cholesterol with no significant effect on high-density lipoprotein (HDL) cholesterol (393,406,408,415,418,420,424). T treatment inhibited triglyceride uptake and lipoprotein lipase activity in abdominal but not femoral subcutaneous adipose tissue depots (407). In several small, randomized, placebo-controlled studies of middle-aged to older men with coronary heart disease, chronic T treatment decreased angina and exercise-induced cardiac ischemia (i.e., increased the time to ST segment depression during exercise testing) (397,402,414,428). Also, acute intravenous administration of T during cardiac catheterization was shown to improve exercise-induced ischemia in older men with CAD, probably by inducing coronary artery vasodilation (411,412,421,422). Subjects with angina also reported significant improvements in quality of life, especially in pain perception and limitation resulting from physical problems (397). Therefore, in contrast to the general perception that androgens are bad for the heart, T administration may have beneficial effects in older men with cardiovascular disease. No clinical trials have studied sufficient numbers of men for long enough periods of time to evaluate the effects of T replacement in older men on major cardiovascular events, such as cardiac death, myocardial infarction, or stroke.

In a recent small controlled study, T treatment improved physical functional status, grip strength, and mood compared to placebo in a small number of relatively frail older men undergoing rehabilitation on a Geriatric Evaluation and Management unit (387). In older men undergoing elective knee or hip arthroplasty, serum T levels were suppressed significantly postoperatively in men treated with placebo, and perioperative supraphysiological T administration tended to improve postoperative strength, physical function, and hematocrit (386). More significant improvements in muscle

strength and physical function, and reduction in hospitalization and rehabilitation duration were probably not observed in this study because the subjects studied were healthy, independently living, older men who were functioning at a relatively high level prior to surgery.

Except for the limited studies mentioned, the effects of T replacement on physical function, health-related quality of life, and the prevention of frailty have not been investigated fully in older men. The prevention of frailty is an important potential goal of T treatment in older men. The frail older population is at high risk for disability, loss of independence, and long-term care, and they utilize a major proportion of health care resources and dollars. Compared with healthy older men, frail older men have lower serum T levels and may derive more functional benefit from T treatment.

Adverse Effects of T Replacement in Studies of Older Men

In controlled studies of up to 3 years in duration, T therapy in older men has been tolerated very well. The only adverse effect that has been found consistently in controlled trials of T treatment in older men is stimulation of excessive erythrocytosis. However, a major caveat is that studies have been powered insufficiently to evaluate the long-term risks of T therapy on prostate and cardiovascular disease, and therefore these potential risks remain unknown.

Stimulation of erythropoiesis and an increase in hematocrit of 3% to 5% over baseline has been found in most controlled trials of T treatment in older men. The development of erythrocytosis (hematocrit over 51%) during T therapy has been reported in 6% to 25% of older men (394,395,399,402,415,416,418). Erythrocytosis has occurred in older men with both parenteral and transdermal T administration, but it has been less common with the latter (397,405). This may be related to the supraphysiological T levels that are produced during the first few days following T ester injections and overall higher mean serum T concentrations compared with the more physiological T levels that are produced with the use of T patches or gel. An excessive increase in hematocrit of more than 55% to 60% results in a substantial increase in blood viscosity and, if untreated, may result in significant hyperviscosity and an increased risk of thrombotic complications. The latter, more serious complications of excessive erythrocytosis have not been observed in T treatment trials in older men.

Although T administration has been reported to induce or worsen obstructive sleep apnea syndrome in younger hypogonadal men (429,430), this complication has not been reported in clinical trials of T therapy in older men (416). Obstructive sleep apnea may be associated with low serum T concentrations (431,432). Therefore, older men with risk factors (e.g., obesity) or symptoms of obstructive sleep apnea (e.g., daytime somnolence and snoring) should be evaluated for symptoms of sleep apnea prior to institution of T treatment and should be monitored for this potentially serious risk during therapy. Validated instruments, such as the Berlin Questionnaire or Epworth Sleepiness Scale, may be used for screening and monitoring (433–436).

The most worrisome potential risk of T replacement ther-

apy in older men is the induction of clinical prostate disease. Most (368,369,437) but not all (371,438,439) descriptive studies have failed to find a significant relationship between endogenous T levels and the development of BPH or prostate cancer. In controlled trials, there has been no significant increase in prostate size, worsening of BPH symptoms, or reduction in urine flow rate in T-treated compared with placebo-treated older men (386,388,399,400,405,406,415,416,418). In some trials of T therapy in older men, serum PSA levels increased slightly, mostly within the normal range (below 4 ng/ml) (392,405,408,416,418), but in most studies, PSA did not change significantly during T treatment. No increase in the need for invasive or surgical therapy for BPH or incidence of clinically apparent prostate cancer has been reported. However, the total number of men treated with T and length of exposure have been limited, and studies have not had the statistical power to evaluate the long-term risks of clinical BPH and prostate cancer in older men treated with T.

In contrast to the suppression of HDL cholesterol that occurs with T replacement in younger men with severe hypogonadism and supraphysiological T administration in young eugonadal men (286,380–385), most controlled trials of T treatment in older men have not found a significant decrease in HDL cholesterol (393,406,415,418,420). As mentioned previously, total and LDL cholesterol decreased in most studies (393,406,418,420) and were unchanged in the remaining trials (394,415) during T therapy of older men. As with prostate disease, clinical trials have not investigated the long-term cardiovascular risk of T replacement treatment in older men. They have not had sufficient statistical power to determine whether T treatment affects the rates of major cardiovascular events such as coronary death, myocardial infarction, and stroke.

Other known adverse effects of T therapy in younger hypogonadal men, such as development or worsening of edema, especially in men with underlying edematous states (e.g., congestive heart failure, hepatic cirrhosis, nephrotic syndrome, and renal failure), gynecomastia, especially in moderate obese men, and suppression of spermatogenesis have not been reported in clinical trials of older men.

APPROACH TO THE DIAGNOSIS OF ANDROGEN DEFICIENCY IN OLDER MEN

Although an increasing proportion of men exhibit low serum T levels with increasing age, a substantial number of older men maintain T levels within the normal range. These individuals may exhibit clinical manifestations consistent with androgen deficiency. It may be argued that they have relative androgen deficiency (i.e., a significant age-related decline in T levels within the wide normal range) contributing to their clinical manifestations. However, the clinical manifestations of androgen deficiency are not specific and are usually multifactorial in nature, and there is no evidence that these men would benefit from T therapy. Furthermore, the clinical significance of slightly low serum T levels in the absence of clinical manifestations consistent with androgen deficiency is not clear. Therefore, the diagnosis of andropause requires both the presence of clinical manifestations or a clinical syndrome, and confirmed serum-free or bio-

available T levels below the normal range for younger men. A rational approach to the diagnosis of androgen deficiency in older men is outlined in Figure 2 and is discussed in the following sections.

The Clinical Syndrome of Androgen Deficiency in Older Men

In order to define a clinical syndrome associated with low T and to identify older men at high risk for low serum T levels, two screening instruments were developed recently. The ADAM questionnaire is a symptom-based instrument that identifies older men with symptoms of low T, with 88% sensitivity and 60% specificity (49). Symptoms associated with low serum T levels in this questionnaire are reduced libido; lack of energy; decreased strength and/or endurance; loss of height; decreased enjoyment of life; feeling sad and/or grumpy; reduced strength of erections; decreased ability to play sports; falling asleep after dinner; and reduced work performance. This symptom complex is similar to that reported by others for the androgen deficiency in older men (440–443). The MMAS questionnaire is an epidemiology-derived instrument that identifies risk factors for low T levels in older men, with 75% sensitivity and 50% specificity (444). Risk factors that are associated with low total T levels include age of 60 years or older; treated diabetes mellitus; treated asthma (a surrogate for glucocorticoid use); sleeplessness (≤ 5 hours); nonsmoking; headaches in the past 2 weeks (a surrogate for stress); low dominance (dislike of a directing role); and body mass index (BMI) 27–30 or >30 .

A combination of symptoms and risk factors may better identify individuals with low T levels. Therefore, a composite of the most prominent clinical manifestations of androgen deficiency in older men is presented in Table 3. Symptoms of androgen deficiency in older men include decreased muscle strength and/or endurance; diminished work and/or athletic performance; loss of height; history of nontraumatic fracture; decreased pubic and axillary hair; reduced physical function; diminished sexual interest and desire; decreased strength and adequacy of erections; fatigue, tiredness, and lack of energy; irritability; depressed mood; decreased general well-being and enjoyment of life; sleep disturbance; sweats; and hot flushes. Signs include decreased muscle mass and strength; increased visceral adiposity; low BMD [osteoporosis or BMD < -2.5 SD below that of young men (T-score < -2.5) or osteopenia (T-score -1 to -2.5)]; vertebral and/or hip fracture; decreased pubic and axillary hair; depressed mood; decreased testis size; gynecomastia; and a normocytic, normochromic anemia.

Repeated Low Serum T Levels in the Absence of Reversible or Remediable Causes

If clinical manifestations suggest androgen deficiency, an early morning (e.g., 8 AM) T level should be measured to confirm low serum T levels. In order to avoid the confounding effects of alterations in SHBG levels on total T, a free or bioavailable T level by equilibrium dialysis or ammonium sulfate, respectively, is recommended for confirmation of androgen deficiency in older men (52,445,446). Alternatively, free or bioavailable T levels may be calculated from

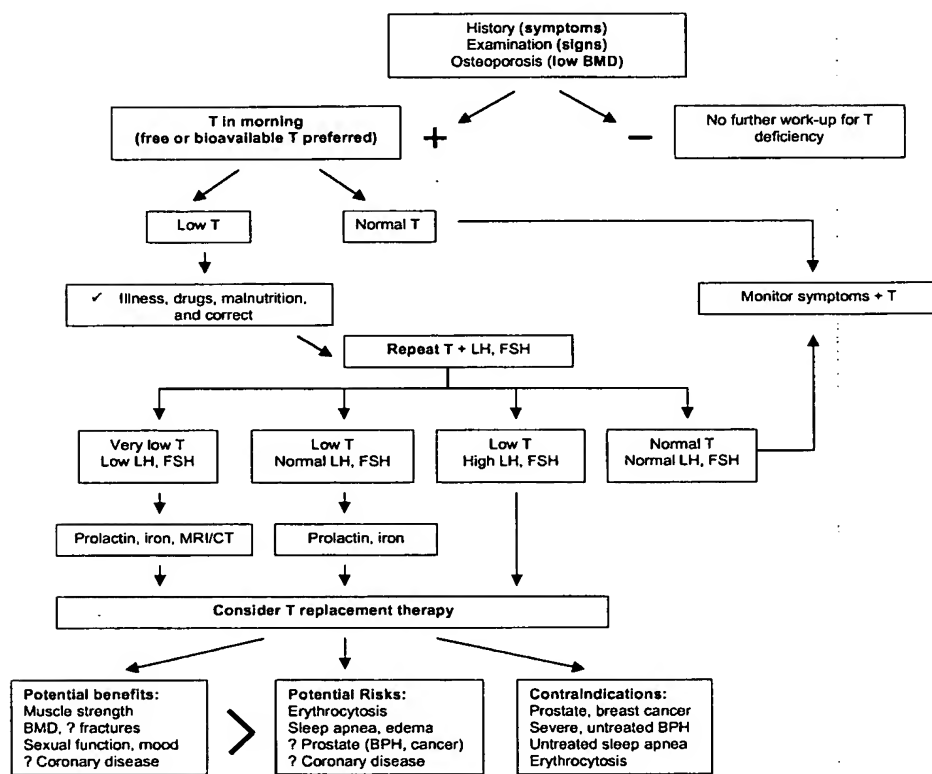


Figure 2. Approach to the diagnosis and treatment of androgen deficiency in older men. Diagnostic evaluation for androgen deficiency should be instituted only in individuals with symptoms and signs consistent with androgen deficiency. Initially, androgen deficiency should be confirmed with the measurement of a morning serum-free or bioavailable T level. Individuals with an initially low free or bioavailable T level should be evaluated for remediable causes of androgen deficiency (e.g., illness, medications, or malnutrition) and have serum T levels repeated in conjunction with gonadotropin (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) levels. Men who demonstrate secondary hypogonadism (low T and normal to low gonadotropin levels) should have measurements of serum prolactin and iron to rule out hyperprolactinemia and hemochromatosis. Imaging of the hypothalamus and pituitary to rule out a tumor or destructive lesion should be reserved for men with very low T and low gonadotropin levels, and/or those with markedly elevated prolactin levels (e.g., >100 ng/ml). T replacement therapy should be considered in men with clinical manifestations of androgen deficiency and repeatedly low serum T levels after appropriate correction or treatment of remediable causes, and if the clinician and patient feel that the potential benefits of treatment outweigh the potential risks, and there are no contraindications. BMD = bone mineral density; MRI/CT = magnetic resonance imaging/computed tomography; BPH = benign prostatic hyperplasia.

total T and SHBG measurements; calculated values are comparable to those measured by equilibrium dialysis and ammonium sulfate precipitation methods (52).

Because total T assays and free T by direct analog immunoassay vary with alterations in SHBG levels, they are not recommended (52,53,55,56,446). However, these assays are usually the only ones that are currently available in local clinical laboratories. If total T or free T using direct analog immunoassay method is used initially to evaluate older men with clinical manifestations of androgen deficiency, values that are in the low-normal to moderately low range (e.g., total T of 350–200 ng/dl) should be confirmed with a free or bioavailable T level measured using a more accurate method. Unless SHBG levels are very low (e.g., nephrotic syndrome), total T levels <200 ng/dl in the presence of consistent clinical manifestations are usually diagnostic of androgen deficiency.

In younger hypogonadal men, T levels below the normal range are usually associated with symptoms of androgen deficiency. Therefore, the normal range in younger men is used as the reference range for older men as well. This approach is analogous to that used for the interpretation of BMD where values are compared to those in younger men (T-score or SD from young controls) and are used to define a clinically significant BMD that is associated with an increased risk of fracture (e.g., osteoporosis defined as a T-score < -2.5).

If the initial serum T level is low, evaluation of underlying acute and chronic illnesses, medications, and nutritional state should be undertaken to determine whether there are reversible or remediable causes of low T levels. In these instances, T level should be repeated after the stress of a current or recent illness is resolved, medications that may lower T (e.g., glucocorticoids or CNS-active drugs) are dis-

Table 3. Androgen Deficiency in Older Men: Clinical Manifestations

Symptoms	Signs
↓ Muscle strength and/or endurance	↓ Muscle mass and strength
↓ Work and/or athletic performance	↑ Visceral adiposity
Loss of height, history of fractures	Low BMD (osteoporosis)
↓ Pubic and axillary hair	Vertebral and/or hip fracture
↓ Physical function	↓ Pubic and axillary hair
↓ Sexual interest and desire	Depressed mood
↓ Strength of erections	↓ Testis size
Fatigue, tiredness, lack of energy	Gynecomastia
Irritability, depressed mood	Normocytic, normochromic anemia
↓ Well-being, enjoyment of life	
Sleep disturbances	
Sweats, hot flushes	

Notes: Composite of symptoms and signs from references (49,438–442). BMD = bone mineral density.

continued, and nutritional compromise (e.g., associated with illness) is corrected.

If no correctable cause of androgen deficiency is found, a T level should be repeated together with serum gonadotropin (LH and FSH) levels. There are significant biological and methodological variations in serum T measurements such that as many as 15% of healthy young men may have a T level below the normal range in a 24-hour period (447). Older men with initially low T values may have normal levels on a subsequent blood sample. Therefore, before committing someone to T replacement therapy, a repeat serum T level should always be obtained to confirm androgen deficiency.

If the initial serum-free or bioavailable T level is within the normal range in a man with symptoms and/or signs of androgen deficiency, the patient's clinical status and serum T levels should be monitored on follow-up visits. Finally, because clinical manifestations of androgen deficiency usually have multiple etiologies in older men, evaluation and appropriate treatment of other causes of clinical findings should be undertaken concomitantly with the work-up for androgen deficiency.

Further Evaluation of Older Men With Low T Levels

If the repeated serum-free or bioavailable T level is low and is associated with inappropriately normal or low gonadotropin levels (i.e., a hormonal pattern of secondary hypogonadism) serum prolactin and iron should be obtained to rule out hyperprolactinemia and hemochromatosis (iron overload), both of which can suppress gonadotropin and T secretion. In individuals with very low T (e.g., total T < 150 ng/dl) and low gonadotropin levels, and/or those with markedly elevated prolactin levels (e.g., >100 ng/ml), an MRI or CT scan of the hypothalamus and pituitary should be performed to rule out a tumor or destructive lesion in this area (448). Men with hyperprolactinemia, hemochromatosis, or hypothalamic-pituitary tumor or destructive lesion should be treated appropriately with consultation from an endocrinologist. In the absence of these conditions, and in older men in whom repeated serum T level is low and is as-

sociated with elevated gonadotropin levels, T replacement treatment should be considered. If the repeat serum-free or bioavailable T and gonadotropin levels are within the normal range, the patient's clinical status should be monitored together with serum T levels.

Consideration of T Treatment in Older Men With Repeatedly Low T Levels

The state of knowledge regarding the benefits and risks of T treatment in older men is based on a limited number of short-term controlled studies (3,449–453). No controlled studies have evaluated the long-term benefits and risks of T therapy in older men. Therefore, there are insufficient data to formulate firm evidence-based clinical guidelines and general recommendations for T therapy in older men. Routine treatment of older men cannot be recommended at this time. After a thorough assessment and discussion of potential benefits and risks of T therapy, individual practitioners must rely on sound clinical judgment and informed patient preferences before deciding to recommend and prescribe T treatment for older men with clinical manifestations consistent with androgen deficiency and repeatedly low serum T levels.

As outlined in Figure 2, T therapy should be considered only in older men with clinical manifestations of androgen deficiency and repeatedly low serum T levels, in whom remediable causes of low T have been corrected or treated appropriately. This approach is consistent with that recommended by consensus panels of experts in the field (446,454). T treatment should be instituted in these men if both the clinician and the patient feel that the potential benefits (e.g., severe muscle weakness, osteoporosis, or alterations in sexual function or mood) of treatment outweigh the potential risks (e.g., erythrocytosis and prostate disease) and if no contraindications exist. Absolute contraindications to T therapy are prostate and breast cancer, and relative contraindications are untreated BPH with severe bladder outlet obstruction, untreated obstructive sleep apnea syndrome, and erythrocytosis.

T THERAPY AND MONITORING IN OLDER MEN

Baseline Evaluation and Goals of T Replacement

Prior to institution of T therapy, a careful baseline clinical evaluation should be performed in order to determine whether there is a history of: prostate or breast cancer, or family history or risks for these androgen-dependent malignancies; severe symptoms of BPH [e.g., using the International Prostate Symptom Score (IPSS) or American Urological Association (AUA) Symptom Score] or sleep apnea (e.g., using the Berlin Questionnaire or Epworth Sleepiness Scale); an abnormal digital rectal examination (e.g., induration or nodule) requiring prostate ultrasound and biopsy; erythrocytosis (hematocrit >52); or an elevated PSA level >4 ng/ml (after empiric treatment for prostatitis and repeat PSA determination).

In the absence of knowledge regarding the dose-response effects of T treatment and lack of evidence for altered androgen requirements in older men, a reasonable goal of T replacement is to restore serum T levels into the mid-normal

range for younger men and to alleviate the clinical manifestations of androgen deficiency.

Preparations Available and Under Development for T Replacement Therapy

There are several formulations available for T replacement therapy in older men (455,456). The most commonly used preparations for T replacement are parenteral 17 beta-hydroxy-esters of T, T enanthate, or cypionate, usually administered by intramuscular injections at a dosage of 150–200 mg every 2 weeks (457). These T esters are safe, effective, and the least expensive formulation available for androgen replacement therapy. However, serum T levels rise to supraphysiological levels during the first few days following injection of T esters and fall into the low-normal range or below normal just before the next injection. The extreme variations in T levels between injections are often accompanied by fluctuations in energy, libido, and mood that may be bothersome to patients. Transient supraphysiological T levels and overall higher average T levels during treatment with T esters may be associated with a higher incidence of side effects, such as erythrocytosis. It is possible that low-dosage androgen supplementation with T enanthate or cypionate (e.g., 50–100 mg every 2 weeks) may have beneficial effects (e.g., on libido, energy, and well-being) without these side effects (458), but this has not been evaluated in controlled clinical trials.

Three transdermal T patches are available for T replacement therapy (459,460). They require daily application but provide more physiological serum T levels (usually in the low- to mid-normal range) than T ester injections. T patches have the advantage that treatment may be discontinued rapidly if adverse effects were to develop in older men, but they are more expensive than replacement with T ester injections. The Testoderm® patch (Alza, Palo Alto, CA) is effective but requires application to a clean, dry, often shaven scrotum, and many men find this site of application unacceptable (218). This patch also produces high serum DHT levels, probably as a result of high 5 alpha-reductase activity in scrotal skin. The clinical consequences of high DHT levels, however, are not known. The Androderm® patch (Watson, Corona, CA) may be applied to non-scrotal skin, but the adhesive and/or enhancing agents used in this patch may cause significant skin irritation (461–463). Skin irritation may be reduced by coapplication of a glucocorticoid cream such as triamcinolone (464). The Testoderm TTS® patch (Alza, Palo Alto, CA) is also applied to non-scrotal skin and causes much less skin irritation because it has less adhesive and does not use enhancing agents (465). However, it is larger than the Androderm patch and may adhere poorly to skin, especially with vigorous exercise or excessive sweating, resulting in lower serum T levels.

Recently, a transdermal T gel, AndroGel® (Unimed/Solvay, Buffalo Grove, IL), became available for T replacement therapy (260,261,466,467). Daily application of this hydroalcoholic gel formulation of T produces physiological serum T levels. The dosage of T gel may be adjusted to achieve T levels in the low-, mid-, or upper-normal range without significant skin irritation. AndroGel may also produce serum DHT levels above the normal range, probably

as a result of the large surface area of skin covered. Transfer of T to partners or children is possible if the skin surface on which T gel is applied is not covered or washed (which is acceptable 1 hour after application).

Oral 17 alpha-alkylated androgens (e.g., methyltestosterone) should not be used for androgen replacement therapy (456). They are less effective than parenteral and transdermal T formulations and are associated with potentially serious hepatotoxicity and greater suppression of HDL cholesterol levels. Outside the United States, an oral T ester formulation, T undecanoate, has been used successfully and safely for many years for T replacement therapy in both young and older androgen-deficient men (449,456,468). Unlike 17 alpha-alkylated androgens, T undecanoate is absorbed directly from the gastrointestinal tract into lymphatics, bypassing initial hepatic inactivation, and it is not associated with hepatotoxicity (468). However, absorption of oral T undecanoate is quite variable and is dependent on co-ingestion of a meal. T undecanoate may become available in the United States in the future. A buccal T formulation is also being developed for androgen replacement therapy. Both the T undecanoate and buccal T have the advantage of rapid withdrawal if adverse effects develop, but the disadvantage of requiring twice daily administration, making compliance more difficult in older patients.

A number of longer-acting T formulations that require injections only every few months are being developed for T replacement therapy (e.g., T undecanoate, T buciclate, and T microspheres), and T implants have been used for androgen replacement outside the United States (456). Although more convenient than currently available T esters, these preparations are probably less suitable for T therapy in older men because rapid withdrawal of androgen would not be possible if adverse effects were to develop during treatment.

Analogous to recently developed selective estrogen receptor modulators used for hormone replacement therapy in post-menopausal women, selective androgen receptor modulators (SARMs) or “designer” androgens are being developed for T replacement therapy. An ideal SARM would be an agent that had all the beneficial effects of T on muscle, bone, sexual function, mood, cognition, and the cardiovascular system without any of the adverse effects on the prostate and cardiovascular system. 7 alpha-methyl-19-nortestosterone (MENT) is synthetic androgen that does not undergo 5 alpha-reduction but is aromatizable to an estrogen. In animal studies, it is approximately 10 times more potent than T in suppressing gonadotropin levels and increasing muscle size, but only two-times more active than T in stimulating prostate growth (469,470). Therefore, it is possible that a low dose of MENT given to hypogonadal men may be able to maintain muscle strength and brain function without stimulating the prostate gland. MENT is being developed as an implant, and preliminary studies suggest that it is able to maintain libido and sexual function in hypogonadal men (471).

Monitoring During T Therapy in Older Men

In older men, clinical examination including a digital rectal examination, hematocrit, and PSA should be repeated 3 and/or 6 months after institution of T treatment, then moni-

tored every 12 months or possibly more frequently thereafter, depending on the clinical status of the patient. Efficacy is determined primarily by subjective and objective clinical responses to T therapy. If symptomatic clinical improvement does not occur in 6–12 months and/or BMD does not improve in 24 months, patients should be re-evaluated and discontinuation of T therapy should be considered. Serum T levels measured at the midpoint of the interval between T ester injections or T patch applications may be useful to confirm that levels are within the midnormal range. Nadir serum T levels (i.e., just before the next T ester injection or patch application) may be helpful in identifying the need for a dosage adjustment.

During T treatment, the following clinical situations require further urological evaluation: development of findings suspicious for prostate cancer on digital rectal examination (e.g., a nodule or induration); PSA level >4 ng/ml that is not complicated by a urinary tract infection (e.g., prostatitis) (472,473); a confirmed increase in PSA of >1.5 ng/ml between two consecutive values over 3–6 months (474); a rate of rise in PSA levels of >0.75 ng/ml/y on sequential values performed over at least 2 years (475); or severe symptoms of BPH (e.g., as assessed using the AUA Symptom Score or IPSS instruments) that are not complicated by medications (e.g., decongestants or antihistamines) or a urinary tract infection (e.g., prostatitis). A urological evaluation including a transrectal ultrasound and prostate biopsy is indicated for an abnormal digital rectal examination or PSA elevation (i.e., a persistent elevation of PSA after empiric antibiotic treatment for prostatitis). Development of an increase in hematocrit $>52\%$ requires reduction in T dosage or discontinuation of therapy. For severe erythrocytosis (e.g., hematocrit $>55\%$), T therapy should be discontinued, and a therapeutic phlebotomy should be performed to acutely reduce the red cell mass and prevent hyperviscosity. After the hematocrit is normalized, T treatment may be reinstituted using a lower dosage or a transdermal formulation. Other causes of erythrocytosis (e.g., obstructive sleep apnea that may be induced or worsened by T treatment, or chronic hypoxic lung disease) should be evaluated and treated appropriately. The development of daytime somnolence, loud snoring, hypertension, edema, and erythrocytosis in an obese older man on T therapy suggests obstructive sleep apnea syndrome. Instruments such as the Berlin Questionnaire or Epworth Sleepiness Scale may be used to assess symptoms of sleep apnea.

SUMMARY AND CONCLUSIONS

In men, there is a gradual and progressive decline in serum T levels with aging that is accentuated by age-associated comorbid illnesses, medications, and malnutrition. Age-related alterations in body composition, sexual function, mood, cognitive function, sleep, and erythropoiesis occur in conjunction with the declining serum T levels. Similar alterations occur in young androgen-deficient hypogonadal men and are improved with T replacement therapy. Therefore, it is reasonable to posit that age-related androgen deficiency may contribute, at least in part, to the changes in physiological function that occur with aging.

Initial short-term controlled studies of T therapy in small

numbers of healthy older men suggest beneficial effects on body composition, BMD, LDL cholesterol, angina, and exercise-induced cardiac ischemia, and possibly muscle strength, libido, general well-being, and certain aspects of cognitive function. In these studies, there have been no significant adverse effects except for erythrocytosis requiring a reduction in dose in some men. Given these findings, it is reasonable to consider T replacement therapy in older men with a clinical syndrome consistent with androgen deficiency and repeatedly low serum-free and bioavailable T levels, in whom the potential benefits of therapy outweigh the potential risks. Because age-related alterations in physiological function are usually a result of multiple etiologies, it is important to evaluate and treat other factors (e.g., inadequate nutritional intake, confounding illness and medication, inactivity or poor conditioning, excessive alcohol, and smoking) in addition to low T levels that may contribute to the clinical syndrome.

A major caveat in treating older men with T is that long-term benefits on fracture incidence, onset of dementia, major cardiovascular outcomes, physical function, frailty and quality of life, and risks of clinical prostate disease (BPH and prostate cancer) and cardiovascular disease are not known. Therefore, routine T treatment of older men cannot be recommended. The balance of benefits and risks of T therapy in older men with low T levels needs to be determined in carefully designed, large, long-term, randomized, placebo-controlled studies. Until the results of these studies are available, practitioners must rely on sound clinical judgment in managing older men with symptoms and signs of andropause. At present, the most prudent course of action is to treat only older men with repeatedly low serum T levels and symptoms and signs consistent with androgen deficiency in whom the potential benefits of therapy clearly outweigh the potential risks, and to carefully monitor treated men for adverse effects. Attention to appropriate exercise and nutrition, and evaluation and treatment of other etiological factors that may contribute to clinical manifestations are essential for optimal management of age-related functional decline in older men.

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Editor Nominations

The Gerontologist

The Gerontological Society of America's Publications Committee is seeking nominations for the position of Editor-in-Chief of *The Gerontologist*, the Society's multidisciplinary journal.

The target start date is July 1, 2002. The Editor-in-Chief makes appointments to the journal's editorial board and develops policies in accord with the scope statement prepared by the Publications Committee and approved by Council (see *The Gerontologist's* General Information and Instructions to Authors). The Editor works with reviewers and has the final responsibility for the acceptance of articles for his/her journal. The editorship is a voluntary position. Candidates must be members of The Gerontological Society of America and dedicated to developing a premier scientific journal.

Nominations and applications may be made by self or others, but must be accompanied by the candidate's curriculum vitae and a statement of willingness to accept the position. **All nominations and applications must be received by March 15, 2002.** Nominations and applications should be sent to the GSA Publications Committee, Attn: Jennifer Campi, The Gerontological Society of America, 1030 15th Street, NW, Suite 250, Washington, DC 20005-1503.

A Review of Androgen-Progestin Regimens for Male Contraception

Review

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It has been 40 years since the administration of steroids to women to prevent pregnancy was approved in many countries, and finally the development of reversible hormonal contraceptives for men seems to be within reach. Surveys performed in recent years suggest that men are willing to share the responsibility of family planning (Glasier et al, 2000, Martin et al, 2000). Worldwide, about 30% of the couples that use contraception use a male method in spite of the evident shortcomings of currently available contraceptives for men. In 1994 at the International Conference on Population and Development, active involvement of men in family planning was given a high priority in the women agenda.

Thanks to public agencies like the World Health Organization (WHO) and the Contraceptive Research and Development Program, the validity of the concept of the hormonal approach to male contraception were proven in large-scale clinical trials. In these studies it was shown that hormones can provide contraceptive protection that promises to be as effective in men as in women (WHO, 1990, 1996). Achievement of azoospermia has been suggested to be the gold standard for this method in order to confer optimal contraceptive protection (WHO, 1990, 1996). Azoospermia achieved with weekly injections of 200 mg of testosterone enanthate (TE) provided a Pearl index (pregnancy per 100 person-years) rating of 0.8 (95% confidence interval [CI] = 0.02–4.5; WHO, 1990). However, various degrees of severe sperm suppression also have been shown to provide acceptable contraceptive protection. When sperm count is suppressed to azoosper-

mia or severe oligozoospermia (sperm count from 0 to $1 \times 10^6/\text{mL}$), contraceptive protection is provided to a Pearl index rating of 1.4 per 100 couple-years (95% CI = 0.4–3.7; WHO, 1996). Therefore, induction of azoospermia or sperm suppression to <1 million/mL can be considered an acceptable contraceptive goal.

Studies performed in the last decade have shown that testosterone (T) administration alone can provide almost universal azoospermia in diverse Asian populations, with minimal side effects (WHO, 1990, 1996). Therefore, phase II and III clinical trials are already being planned with androgen regimens in the Asian population. The encouraging preliminary results of these studies offer some promise that an androgen-alone contraceptive may be on the market in those countries within the next few years (Zhang et al, 1999).

Testosterone-alone regimens are not as effective in the Caucasian population as they are in the Asian population, but small-scale clinical trials performed over the last few years have suggested that if a progestin is added to the androgen, profound suppression of sperm production can be achieved in Caucasian men (Meriggiola and Bremner, 1997).

In spite of the fact that all of these studies have shown that hormonal contraception for men is feasible and effective, the lack of involvement of drug companies has prevented the transit of these concepts from small pilot trials to large-scale studies aimed at the development of suitable products for the market. In 1997, a group of leading researchers promulgated a manifesto on male contraception. The aim of this manifesto was to sensitize drug companies, politicians, and research foundations "to commit themselves to the development of male contraception for the sake of future generations" (Nieschlag and Behre, 1998). This appeal was finally received and in November 2002 when 2 major pharmaceutical companies committed themselves to the development of a hormonal contraception for men (Schering AG, 2002).

Large-scale clinical trials are now being planned with various combinations of progestins and androgens to confirm and extend the preliminary results obtained in small pilot studies and to eventually test the contraceptive effectiveness of these combinations. Advantages of this hormonal combination include the vast experience of years of clinical use of both classes of compounds and their relatively low cost.

The purpose of this article is to review the literature

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Table 1a. Number of various androgen-progestin regimens tested in papers from 1960 to 2002*

	T Oral†	TTS	TE	TP	T Pellets	Tpc/DHT	TC	TU (IM)	19 T	Total Regimens	Total Papers
CPA	1	...	6	7	4
DMPA	15	1	1	...	6	...	2	25	14
MPA	4	2	6	3
DSG‡	...	3	6	...	6	15	6
LNG‡	1	3	7	4	...	2	...	17	10
NET/E/A	1	3	...	4	...	8	4

* T indicates testosterone; TTS, transdermal testosterone; TE, testosterone enanthate; TP, testosterone propionate; Tpc, percutaneous testosterone; DHT, dihydrotestosterone; TC, testosterone cypionate; TU, testosterone undecanoate; CPA, cyproterone acetate; DMPA, depot medroxyprogesterone acetate; MPA, medroxyprogesterone acetate; DSG, desogestrel; LNG, levonorgestrel; NET, norethisterone; NETA, norethisterone acetate; NETE, norethisterone enanthate.

† T Oral includes testosterone undecanoate and methyl-testosterone.

‡ Includes both oral and implants.

on androgen-progestin combinations, to understand lessons learned from these trials, and to determine how these results can be applied to the design of large multicenter clinical trials with the ultimate goal of developing an optimal hormonal male contraceptive.

Methods

This review includes all studies reporting the outcome of androgen-progestin regimens, in terms of sperm suppression, published in the peer-reviewed literature between 1960 and September 2002. For this review, the database MEDLINE was searched. English-only publications were included in the search, and subsequent bibliographies were cross-referenced. Where more than 1 publication reported the same study, the data were analyzed only once. For a detailed breakdown of the various studies, please refer to the tables. Because of the apparent ethnic differences in responsiveness to steroids, results from clinical trials of potential hormonal contraceptive regimens have been analyzed and reported separately in the Asian and Caucasian populations. Healthy subjects of all ethnic groups, within an age range of 21–50 years and with normal seminal parameters, were included in the studies. Only studies in which hormone administration lasted longer than 12 weeks were included in the analyses. Maximal suppression of sperm count (rate of azoospermia) was considered for all studies. In most of the papers it coincided with the end of the study, but in many papers it could not be detected at which

time of the study maximal sperm suppression occurred. Only rate of azoospermia is considered for analysis in this paper, since the degree of sperm suppression was not always clearly reported in the results sections of the various studies. Gonadotropin suppression could not be considered in this review, since the different gonadotropin assays and their evolution throughout the years would not allow for a comparison among the studies.

Statistical Analyses

The rates of azoospermia observed in each study were plotted according to the regimen, and the mean rate of azoospermia was calculated. The total number of azoospermic men, as well as the total number of treated cases, were also reported in the text of the figures. These numbers were used for the comparison between different regimens by evaluating the Yates corrected chi-squared test and the odds ratio (OR) together with the 95% CI. Statistical evaluations were performed by the SPSS/PC+ statistical package version 5.0 (Dixon et al, 1990; Norusis, 1992).

Mechanisms of Hormonal Suppression of Male Fertility

The mechanism(s) by which gonadal steroids inhibit male fertility is the suppression of sperm production achieved through inhibition of gonadotropin secretion. The various progestins have different antigonadotropic potency: 19-nortestosterone derivatives have a stronger suppressive

Table 1b. Number of subjects treated with the different regimens*

	T Oral†	TTS	TE	TP	T Implants	Tpc/DHT	TC	TU (IM)	19 T	Total Men
CPA	8	...	36	44
DMPA	136	6	10	...	62	...	57	271
MPA	23	13	36
DSG‡	...	17	45	...	100	162
LNG‡	4	11	90	59	...	30	...	194
NET/E/A	13	28	...	54	...	95

* T indicates testosterone; TTS, transdermal testosterone; TE, testosterone enanthate; TP, testosterone propionate; Tpc, percutaneous testosterone; DHT, dihydrotestosterone; TC, testosterone cypionate; TU, testosterone undecanoate; CPA, cyproterone acetate; DMPA, depot medroxyprogesterone acetate; MPA, medroxyprogesterone acetate; DSG, desogestrel; LNG, levonorgestrel; NET, norethisterone; NETA, norethisterone acetate; NETE, norethisterone enanthate.

† T Oral includes testosterone undecanoate and methyl-testosterone.

‡ Includes both oral and implants.

Table 1c. Number of subjects that became azoospermic with the different androgen-progestin regimens*

	T Oral†	TTS	TE	TP	T Implants	Tpc/DHT	TC	TU (IM)	19 T	Total Azoospermic Men	Total Men in Studies	% Azoospermic
CPA	1	...	22	23	44	52
DMPA	108	4	9	...	26	...	50	197	271	73
MPA	0	8	8	36	22
DSG‡	...	7	36	...	79	122	162	75
LNG‡	0	2	53	12	...	13	...	80	194	41
NET/EIA	7	19	...	49	...	75	95	79
Total	8	9	219	4	88	39	26	62	50	505	802	63

* T indicates testosterone; TTS, transdermal testosterone; TE, testosterone enanthate; TP, testosterone propionate; Tpc, percutaneous testosterone; TC, testosterone cypionate; TU, testosterone undecanoate; CPA, cyproterone acetate; DMPA, depot medroxyprogesterone acetate; MPA, medroxyprogesterone acetate; DSG, desogestrel; LNG, levonorgestrel; NET, norethisterone; NETA, norethisterone acetate; NETE, norethisterone enanthate.

† T Oral includes testosterone undecanoate and methyl-testosterone.

‡ Includes both oral and implants.

effect on gonadotropins compared with the progesterone-derived progestins.

The mechanism by which progestins suppress gonadotropins is still unclear. Although previous studies attributed the antigonadotropic effect of progestin to their androgenic activity, more recently a possible direct inhibitory effect of these compounds on gonadotropin secretion has been postulated (McEwen et al, 1983; Couzinet et al, 1996). The direct inhibitory effect of progestins on gonadotropins, independent of estrogens and androgens, could also explain the additive and synergistic effects of this compound when combined with other steroids, in this case androgens. Progesterone receptors have been found both at the hypothalamus and pituitary level in rats and nonhuman primates.

After exogenous administration of sex hormones such as androgens, progestins, or estrogens, Leydig cells decrease in volume; consequently, serum T production also decreases (Flickinger, 1977a, b). T levels fall below physiological levels both intratesticularly and in the peripheral circulation (Morse et al, 1973; McLachlan et al, 2002). Therefore, administration of androgens or androgen-like substances to re-establish peripheral physiological T levels is mandatory for non-T-alone hormonal contraceptive regimens that act through this mechanism to maintain androgen-dependent physiological functions. As a result of FSH and intratesticular T suppression, studies in both animals and humans have demonstrated that after hormone administration, sperm development stops at the pachytene spermatocyte stage, whereas degeneration of later stage spermatids occurs (Termer and MacLaughlin 1973; Hikim et al, 1995). This mechanism maintains spermatogonia and thus implies the potential reversibility of the contraceptive method. According to the degree of T depletion from the testis, remaining spermatids and spermatocytes may complete their development or slough from the seminiferous epithelium. This means that hormone regimens require some time to be fully effective over the human spermatogenic cycle of about 70 days. The mean time reported to induce azoospermia ranged from 6 to 12 weeks in most clinical trials.

After stopping hormone administration, gonadotropin secretion recovers, resulting in full resumption of Leydig cell function and spermatogenesis. The time for spermatogenesis to resume normal levels has varied widely among different studies, ranging from a few weeks to several months, as the result of the long-lasting effect of some steroids, which may accumulate in the subcutaneous tissue. However, it should be emphasized that in all studies performed so far, full recovery of spermatogenesis was always achieved in all subjects. This characteristic gives to hormonal male contraception an important advantage over other currently available forms of contraception such as vasectomy.

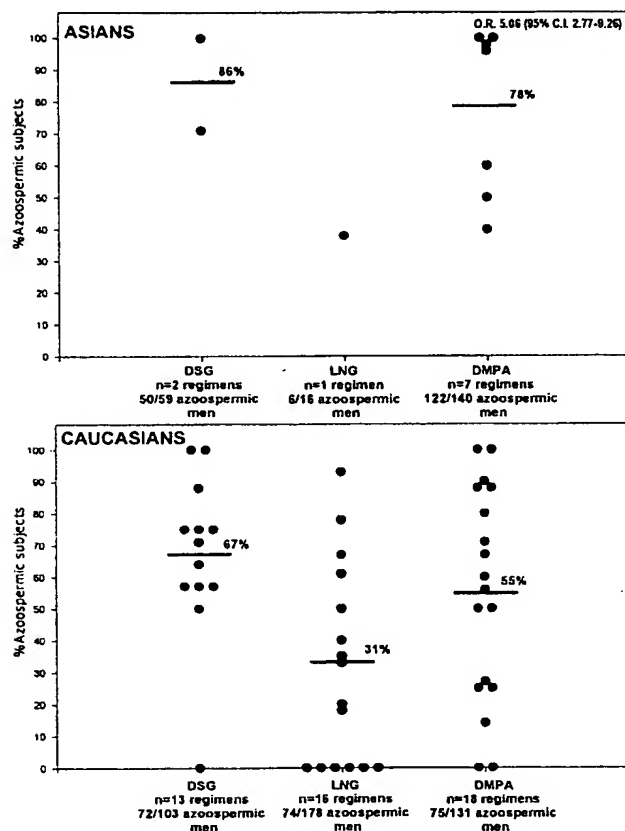


Figure 1. Rates of azoospermia of androgen-progestin regimens in the Asian and Caucasian populations. Full circles indicate rates of azoospermia achieved with each different androgen-progestin regimen in the two populations, respectively. Regimens were divided according to the progestin that was administered in combination with injectable testosterone (T). Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing Asians vs Caucasians.

Rationale for the Androgen-Progestin Combinations

The rationale for combining androgens with progestins to suppress fertility in men is based on the additive and synergistic effects that these 2 steroids have on the suppression of gonadotropins, and thus spermatogenesis. Previous studies have demonstrated that when given alone at doses that are safe for administration, currently available progestins do not induce profound suppression of gonadotropins, and thus spermatogenesis, in men (Johansson and Nygren, 1973; Morse et al, 1973; Koch et al, 1976; Roy et al, 1976; Fredricsson, 1978; Fogh et al, 1979; Moltz et al, 1980; Wang and Yeung, 1980; Fredricsson and Carlstrom, 1981; Kamischke et al, 2000a). Additionally, androgens alone, even when administered at high, supraphysiological doses, do not uniformly induce a degree of spermatogenic suppression sufficient for contra-

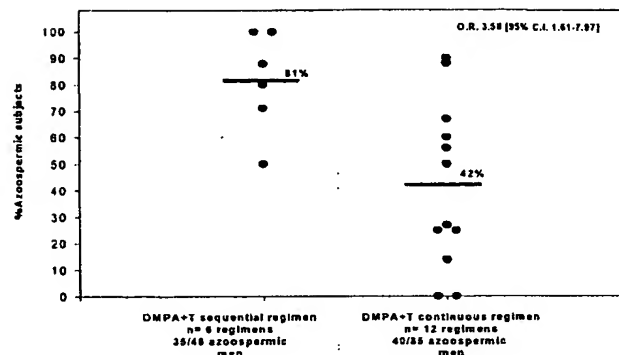


Figure 2. Rates of azoospermia following DMPA plus testosterone (T) administered in a sequential or combined-continuous fashion. Full circles indicate rate of azoospermia achieved with each different regimen. In both groups the maximal sperm suppression was reported. Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing sequential vs combined-continuous regimens. See text and Table 2 for details.

ception (WHO, 1990, 1996; Matsumoto, 1990). Moreover, high T levels may induce changes in some hematocrit and lipid parameters that may have potential long-term adverse effects (Bagatell et al, 1994; Meriggiola et al, 1995). The doses of androgen and progestin can be adjusted to achieve maximal gonadotropin suppression and, at the same time, avoid induction of supraphysiological serum androgen levels, thereby improving the safety of the regimen.

In addition to the effects at the hypothalamus-pituitary

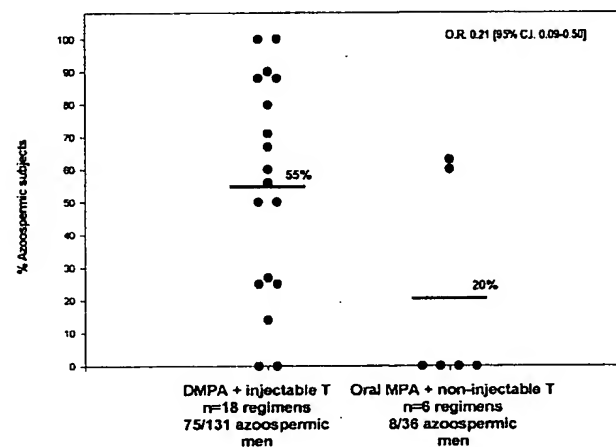


Figure 3. Rates of azoospermia following DMPA injections in combination with injectable testosterone (T: testosterone enanthate [TE], testosterone propionate [TP], T pellets, testosterone cypionate [TC], and 19-nortestosterone [19-NT]) or medroxyprogesterone acetate (MPA) oral in combination with noninjectable T (oral or transdermal). Full circles indicate rate of azoospermia achieved with each different regimen. Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing noninjectable vs injectable T regimens. See text and Table 3 for details.

Table 2. Rates of azoospermia with medroxyprogesterone acetate and various testosterone formulations*

Medroxyprogesterone Acetate	Methyl Testosterone (Oral)		Testosterone/DHT (Percutaneous)		References
	Dose	Azoospermia (%)	Dose	Azoospermia (%)	
20 mg/d	20 mg/d	0	Bain et al, 1980
20 mg/d	10 mg/d	0	Bain et al, 1980
20 mg/d	50/100 mg/T	60	Soufir et al, 1983
20 mg/d	125 mg DHT/250 mg T	62.5	Guerin et al, 1988
10 mg/d	10 mg/d	0	Bain et al, 1980
5 mg/d	10 mg/d	0	Bain et al, 1980

* T indicates testosterone; DHT, dihydrotestosterone.

level, there are studies that indicate the possibility of a direct inhibitory role of the progestins at the testicular level. A number of studies in animals suggest that progestins may affect spermatogenesis by exerting a direct effect at testicular and posttesticular levels. A variety of mechanism(s) have been suggested to explain this observation. Progestins may reduce the androgen concentrations within the testis by reducing androgen biosynthesis, altering androgen metabolism, and/or acting directly at the receptor level by competitive binding, thereby reducing the effective level of intratesticular androgens (Lobl et al, 1983; Mauvais-Jarvis et al, 1974; Vreeburg et al, 1976; Worgul et al, 1979). Studies in animals suggest that various changes in epididymal structures occur, although their significance remains unclear (Srivastava and Malaviya, 1980; Srivastava, 1981). It should be noted that the above described effects have only been reported in vitro and in vivo at high progestin concentrations, and their relevance to the doses necessary for contraception in men is unclear. The importance of the contribution of local effects relative to the overall contraceptive effects of this regimen is still unknown.

Studies With Androgen-Progestin Combinations

Medroxyprogesterone Acetate—Medroxyprogesterone acetate (MPA) was developed at the end of the 1950s, and in 1960 the U.S. Food and Drug Administration approved its use for regulation of menstrual disorders. Since then a vast amount of literature has been published on this progestin, which is used in the treatment of a wide variety of conditions and for contraception in women. In men, MPA has been used for the treatment of prostatic hypertrophy and hypersexuality and in experimental male contraception.

Over the last 40 years, 17 papers were published describing studies in which 31 different regimens using MPA were administered to healthy men for suppression of spermatogenesis (Table 1a). These regimens consisted of either the oral or injectable form of MPA given in combination with different T formulations. A total of 307 men completed at least 12 weeks of hormone administra-

tion (Table 1b). Altogether, 205 (67%) subjects achieved azoospermia (Table 1c, Figures 1 through 3). Three of these studies were performed in Asian populations (Pangkahila, 1991; Lee et al, 1979; WHO, 1993).

Like all other hormonal regimens tested so far, the rates of azoospermia achieved in the Asian population varied widely but were generally higher than that achieved in the Caucasian population (Figure 1). One hundred twenty-two of the 140 Asian subjects (87%) achieved azoospermia, whereas only 75 of the 131 (57%) Caucasian subjects became azoospermic ($P < .001$). Preliminary studies have hypothesized that genetic factors such as a different sensitivity of the pituitary or of the testis to the suppressive effects of steroids could be present between the Caucasian and Asian population (Suhana et al, 1999). Another interesting hypothesis is a difference in the diet, which can result in a different steroid metabolism (Handelsman et al, 1995; Johnson et al, 1998; Lookingbill et al, 1991; Santner et al, 1998; Sinha et al, 1998; Wang et al, 1998). Whether this different sensitivity to the suppressive effects of steroids will still be confirmed in future studies, the mechanism needs to be thoroughly investigated.

In the Caucasian population, the oral and the depot injectable MPA formulations were administered in combination with oral or injectable T preparations, respectively. Oral MPA was given in combination with oral T, such as methyl-T, or with percutaneous dihydrotestosterone (DHT; Tables 1a and 2; Bain et al, 1980; Guerin and Rollet, 1988; Soufir et al, 1983). Depot medroxyprogesterone acetate (DMPA) injected every 4–6 weeks was given in combination with TE (Alvarez-Sanchez et al, 1977; Brenner et al, 1977; Frick et al, 1977a; Melo and Coutinho, 1977; Alvarez-Sanchez et al, 1979; Faundes et al, 1981; Frick et al, 1982; Wu and Aitken, 1989; Pangkahila et al, 1991; WHO 1993); testosterone propionate (TP; Frick et al, 1977a); testosterone cypionate (TC; Paulsen et al, 1980; Lee et al, 1979); 19-nortestosterone (19-NT); or T pellets (Knuth et al, 1989; Handelsman et al, 1996; WHO 1993; Tables 1a and 3). Oral combinations

were less effective than injectable DMPA (Figure 3) in the suppression of spermatogenesis ($P \leq .001$). After administration of DMPA plus injectable T, 57% (75 of 131) of the volunteers achieved azoospermia compared with 22% azoospermic (8 of 36) of the men who received the oral preparation of MPA (Figure 3).

DMPA plus injectable T was administered in a combined-continuous or sequential fashion (Alvarez-Sanchez et al, 1977, 1979; Frick et al, 1977b; Faundes et al, 1981; Frick et al, 1982; Knuth et al, 1989; Table 2). Sequential regimens included an initial period during which the 2 steroids were administered at higher doses, followed by a second phase in which lower steroid doses were administered. With the sequential regimens, profound sperm suppression was achieved by means of the high doses of DMPA and T. Sperm suppression achieved in this phase was more profound than that achieved with the combined-continuous regimens ($P \leq .012$; Figure 2). Thirty-five of 46 subjects (76%) became azoospermic after an initial phase of high-dose hormone administration, whereas only 40 of 85 subjects (47%) achieved azoospermia at the end of a combined-continuous DMPA plus T administration (Figure 2). However, in all studies the regimens used in the second phase failed to maintain sperm suppression, and spermatogenesis recovered in most of the subjects in all studies. No major adverse effects were reported with any of these regimens.

Cyproterone Acetate—Cyproterone acetate (CPA) is a synthetic steroid with both progestational and antiandrogenic properties (Neumann and von Berswordt-Wallrabe, 1966; Steinbeck et al, 1971). Because of this combination of activities, CPA has been used in conditions in which a profound suppression of androgen activity is needed in men (eg, hypersexuality or prostate cancer). During these treatments, a decrease of gonadotropin and T levels and a dramatic reduction in sperm production was observed. These observations prompted researchers to test the possibility that CPA could be used for suppression of male fertility. Ten papers have been published in which CPA was given at doses ranging from 200 to 5 mg/d, orally, to test its effectiveness in suppressing spermatogenesis and gonadotropins (Petry et al, 1972; Morse et al, 1973; Koch et al, 1976; Roy et al, 1976; Fredricsson, 1978; Fogh et al, 1979; Roy and Chatterjee, 1979; Moltz et al, 1980; Wang and Yeung, 1980; Fredricsson and Carlstrom, 1981). These studies were also stimulated by the promising results obtained in animals, which suggested that CPA could inhibit fertility through a direct effect at the posttesticular level. Among 76 men who received CPA for 16–26 weeks, azoospermia was only occasionally achieved in a few men and not consistently maintained. In most of the men, sperm suppression was variable and exhibited various degrees of oligozoospermia. In these trials, a decrease of sperm motility and normal morphol-

ogy was reported. Whether these changes, probably due to a direct effect of CPA at posttesticular level, lead to a significant reduction of fertility potential of these spermatozoa is unclear. However, major side effects of this regimen included a decrease of both libido and sexual potency that was so severe that, in many cases, volunteers were unable to produce an ejaculate for semen analysis.

In 1983, Lohiya and Sharma proposed to combine CPA with T in order to avoid side effects due to androgen depletion. They administered CPA 1 mg/kg IM and TE 2 mg/kg IM every 15 days over a period of 90 days to male Langur monkeys. All animals became azoospermic, and no significant changes of any biochemical parameters were detected. Two years later, the same promising results were reported after administration of 20 mg/d of CPA in combination with 250 mg/fortnight of TE to 6 men (Roy and Prasad, 1985). Five of 6 men became azoospermic, and 1 man had a sperm count <1 million/mL after 20 weeks of administration. In spite of these promising preliminary data, this hormonal combination did not receive further attention, primarily for 2 reasons: 1) these data in men were never published in a scientific peer-reviewed journal, and 2) it seemed somehow illogical to combine an androgen with an antiandrogen. This regimen was explored again 10 years later. The proposed rationale for testing this hormonal combination was based on the peculiar combination of progestogenic and antiandrogenic activity of the progestin. CPA may suppress gonadotropins because of its progestogenic activity as well as act at the testicular and posttesticular level, blocking the stimulatory effect of residual or exogenous intratesticular T on sperm development as the result of its antiandrogenic activity. The lack of any suppressive effect of CPA on plasma sex hormone binding globulin (SHBG) levels could contribute to the avoidance of increased free T levels, which is different than other progestins like levonorgestrel (LNG), which is known to decrease SHBG levels (Meriggiola et al, 2002b).

Four studies have been published (Meriggiola et al, 1996, 1997, 1998, 2002b) in which CPA was given at doses of 100, 50, 25, 12.5, and 5 mg/d in combination with TE 100 or 200 mg/wk or with oral testosterone undecenoate (TU) 160 mg/d to 44 subjects (Table 4). Overall, 23 (52%) subjects became azoospermic, 14 (32%) severely oligozoospermic (<1 million/mL), and in 3 (7%) subjects the sperm count remained above 3 million/mL after 16 weeks of hormone administration (Figure 4a). When the same dose of CPA (25 mg/d) was administered with an oral (TU) or injectable (TE) androgen, the regimen in which oral T was administered seemed to be less effective than that in which IM TE injections were given. One of 8 subjects achieved azoospermia with the oral combination vs 4 of 5 subjects in the CPA plus TE regimen (Figure 4a). Comparing gonadotropin levels be-

Table 3. Rates of azoospermia with depot medroxyprogesterone acetate and various testosterone formulations

Depot Medroxy-progesterone Acetate	Testosterone Elanathate (IM)		Testosterone Propionate (IM)	
	Dose	Azoospermia (%)	Dose	Azoospermia (%)
Sequential regimens				
1000 mg × 1 then 300 mg/mo	250 mg/mo	71
1000 mg × 2 then 150 mg/mo	250 mg/mo	88
1000 mg/mo then 75 mg/biweek	250 mg/biweek	100
1000 mg/mo then 150 mg/mo	500 mg/mo	100
1000 mg × 1 then 150 mg/mo	500 mg/mo	80
400 mg/mo
250 mg/6 wk	200 mg/wk for 6 wk then every 3	96
250 mg/6 wk
250 mg/6 wk
Combined-continuous regimens				
300 mg single dose
200 mg/mo	250 mg/mo	100
200 mg/mo	250 mg/mo	60
200 mg/mo
200 mg/mo
200 mg/mo
150 mg/mo	100 mg/mo	50
150 mg/mo	200 mg/mo	25
100 mg/mo	100 mg/mo	100
100 mg/mo	200 mg/mo	14
100 mg/mo
100 mg/mo	4 capsule impl. at 1° inj.	67
100 mg/mo	100 mg/mo	0
100 mg/mo	250 mg/mo	0
100 mg/mo	250 mg/mo	88
50 mg/mo

tween the 2 regimens, it is evident that reduced gonadotropin suppression was achieved with the oral combination (Figure 4b). Although the studies included a very small number of subjects, it would appear that the less profound gonadotropin suppression achieved with oral T may be due to the inconsistent serum T levels achieved after oral TU intake, which may not be able to maintain gonadotropin, and hence sperm suppression (Meriggiola et al, 1997). No major side effects were detected with this regimen. No changes of sexual function or behavior were reported, with the exception of a significant decrease of morning erections in the group that received the highest dose of CPA (100 mg/d). There was a decrease of red cell parameters in all groups, which seemed to be dependent on the dose of CPA and was probably related to its antiandrogenic activity. Further large-size clinical trials may be required to test the safety and efficacy of low doses of CPA with T in multiethnic groups of men.

19-Nor-Progestins—Based on animal studies and clinical studies in women, 19-norderived progestins are

known to be potent in terms of gonadotropin suppression (Couzinet et al, 1996). Among this class of steroidal compounds are norethisterone (NET), norethynodrel, and its dextrorotatory isomer LNG (ie, the biologically active form of this progestin). The progestins of this class are known to be potent suppressors of gonadotropin secretion, and when administered to men these compounds induced a profound suppression of sperm production (Frick, 1973). However, a decrease of libido and sexual potency was also reported, presumably due to the suppression of T production secondary to gonadotropin suppression (Kamischke et al, 2000b). Therefore, like other progestins available thus far, nor-progestins should not be administered alone for male contraception because their residual androgenic activity is not sufficient to maintain androgen-dependent physiological functions like libido or sexual potency (Kamischke et al, 2000a). Also, due to their estrogenic activity or to inadequate androgen replacement, administration of these progestins resulted in a high rate of gynecomastia (Paulsen et al, 1962; Kuhn et al, 1997).

Table 3. *Continued*

T Pellets (Subdermal)		Testosterone Cypionate (IM)		19-Nortestosterone (IM)		Reference
Dose	Azoospermia (%)	Dose	Azoospermia (%)	Dose	Azoospermia (%)	
...	Alvarez-Sanchez et al, 1977, 1979
...	Alvarez-Sanchez et al, 1977, 1979
...	Frick et al, 1982
...	Frick et al, 1982
...	Faundes et al, 1981
...	...	200 mg/mo	50	Lee et al, 1979
...	WHO, 1983
...	200 mg/wk for 6 wk then every 3 wk	42	Knuth et al, 1989
...	200 mg/mo for 6 wk then every 3 wk	98	WHO, 1983
800 mg	90	Handelsman et al, 1996
...	Pangkahlla et al, 1991
...	Wu et al, 1989
...	...	250 mg/mo	56	Paulsen et al, 1980
...	...	200 mg/mo	60	Lee et al, 1979
...	...	400 mg/mo	40	Lee et al, 1979
...	Frick et al, 1977a
...	Brenner et al, 1977
...	Pangkahlla et al, 1991
...	Brenner et al, 1977
...	...	250 mg/mo	27	Paulsen et al, 1980
...	Frick et al, 1977a
...	Frick et al, 1977a
...	Frick et al, 1977a
...	Melo et al, 1977
...	...	250 mg/mo	25	Paulsen et al, 1980

Table 4. *Rates of azoospermia with cyproterone acetate and various testosterone formulations*

Cyproterone Acetate (Oral)	Testosterone Undecanoate (Oral)		Testosterone Enanthate (IM)		References
	Dose	Azoospermia (%)	Dose	Azoospermia (%)	
100 mg/d	100 mg/wk	100	Meriggiola et al, 1996
50 mg/d	100 mg/wk	100	Meriggiola et al, 1996
25 mg/d	160 mg/d	12.5	Meriggiola et al, 1997
25 mg/d	100 mg/wk	80	Meriggiola et al, 1998
12.5 mg/d	100 mg/wk	60	Meriggiola et al, 1998
5 mg/d	100 mg/wk	56	Meriggiola et al, 2002
...	200 mg/wk	0	...

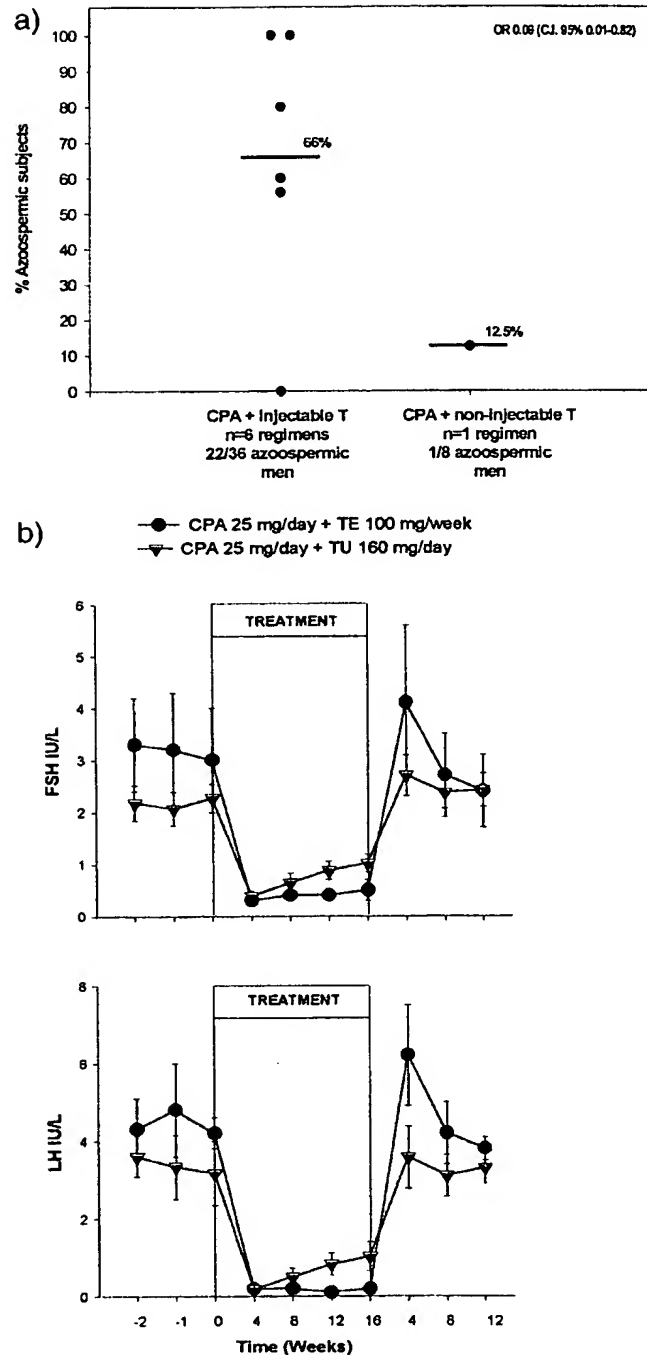


Figure 4. (A) Rates of azoospermia following administration of cyproterone acetate (CPA) plus testosterone enanthate (TE) injections or oral testosterone undecenoate (TU). Full circles indicate rate of azoospermia achieved with each different regimen. Lines represent mean of the azoospermic rate in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing noninjectable vs injectable testosterone (T) regimens. See text and Table 4 for details. (B) Mean serum FSH and LH levels after oral intake of CPA 25 mg/d plus weekly injections of 100 mg TE or plus oral intake of TU 160 mg/d throughout the study periods. See text and Table 4 for details.

These progestins have therefore been tested in combination with different androgens. Of this group of compounds, LNG and NET, norethisterone enanthate (NETE) and norethisterone acetate (NETA) were the most commonly used progestins in clinical trials. Levonorgestrel seemed to be most attractive because of its potency and the large number of formulations in which it is available for women (oral, injectable, transdermal, subcutaneous, intravaginal, and intrauterine), offering the prospect of different routes of administration for men as well. Ten trials (Fogh et al 1980a, b, c; Bebb et al, 1996; Anawalt et al, 1999; Buchter et al, 1999; Gao et al, 1999; Kamischke et al, 2000b; Pollanen et al, 2001; Gonzalo et al, 2002) have been published in which LNG was administered to normal healthy men to test the effects on sperm suppression (Table 1a). Oral LNG at doses ranging from 125 to 500 $\mu\text{g/d}$ or LNG implants were given in combination with TE or TU injections, oral T or transdermal T, or DHT for at least 6 months (Table 5). Overall, of the 194 subjects treated with these hormonal combinations, 80 (41%) achieved azoospermia (Tables 1b and c; Figure 5). As with other progestins, a combination of oral or transdermal T/DHT with oral LNG (Fogh et al, 1980c; Buchter et al, 1999; Pollanen et al, 2001) tended to be less effective in terms of sperm suppression compared with oral LNG given in combination with injectable T formulations ($P < .001$; Figure 5; Fogh et al, 1980a, b; Bebb et al, 1996; Anawalt et al, 1999; Gao et al, 1999; Kamischke et al, 2000b; Gonzalo et al, 2002). Fourteen of 74 subjects (19%) who received oral or implant LNG plus oral or transdermal T/DHT became azoospermic compared with the 66 of 120 subjects (55%) that became azoospermic after oral LNG plus injectable TE or TU (Figure 5).

Four papers have been published in which NET, NETE, or NETA were administered to 95 subjects for 6–9 months (Tables 1a through c). In these studies, 79% (75 subjects) became azoospermic (Guerin and Rollet, 1988; Lobel et al, 1989; Kamischke et al, 2001, 2002; Table 6). The combined administration of injectable NETE plus TU was more effective in sperm suppression (91% azoospermic, 49 of 54 subjects) compared with the oral administration of NETA plus oral TU or percutaneous T (63% azoospermic, 26 of 41; $P \leq .001$; Figure 6).

The progestins of this class retain varying degrees of androgenic activity that possibly accumulate with that of the exogenous androgen. The reported decrease of SHBG levels and displacement of T from its binding sites caused by these progestins may further contribute to increased free T levels (Pugeat et al, 1981; Darney, 1995). Together, these characteristics may explain the fact that the adverse effects induced by most of the 19-norsteroid progestin when combined with androgens are identical to those reported with high-dose androgens, in-

cluding weight gain, acne, and a decrease in high-density lipoprotein (HDL) cholesterol. Further large-scale studies are required to determine both efficacy and safety of injectable TU in combination with NETE or LNG in multiethnic settings.

Desogestrel—Since the intrinsic androgenicity of progestins like LNG or NET/E/A was thought to contribute to the androgen-related side effects observed with these progestins, the reduction of the androgenic activity in the third generation progestins was thought to provide advantages in terms of safety and possibly of sperm suppression. Six papers (Wu et al, 1999; Anawalt et al, 2000; Kinniburgh et al, 2001; Morton Hair et al, 2001; Kinniburgh et al, 2002; Anderson et al, 2002; Table 7) have been published in which the progestin desogestrel (DSG) at doses of 75, 150, or 300 $\mu\text{g/d}$ or as an implant releasing 68 mg/d of etonogestrel was combined with TE 100 or 50 mg/wk or with 400-mg T pellets (Table 1a). Overall, 162 subjects received 1 of these regimens for 24 weeks. Azoospermia was achieved in 75% of the subjects (122 of 162; Tables 1b and c; Figure 7). With DSG 300 μg plus TE 100 mg/wk, 13 of 16 subjects (81%) became azoospermic, whereas when the dose of TE was reduced to 50 mg/week, azoospermia was achieved in 8 of 8 subjects (100%). Lower doses of DSG (150 $\mu\text{g/d}$) in combination with 100 or 50 mg/wk TE induced azoospermia in 79% (11 of 14) and 57% (4 of 7) of the subjects, respectively. In one study (Wu et al, 1999) when TE administration was delayed for 3 weeks to allow for the suppression of gonadotropins and depletion of intratesticular T concentrations, no difference in sperm suppression was found compared with the same regimen (DSG 300 $\mu\text{g/d}$ plus TE 100 mg/wk) administered from the beginning: percentage of azoospermia was 75% (6 of 8) vs 88% (7 of 8), and time to azoospermia was 14 ± 2.7 weeks in the 2 groups, respectively. Both studies with DSG and TE reported a decrease of HDL cholesterol that seemed to be dependent on the dose of both the progestin and the androgen (minimum DSG 150 $\mu\text{g/d}$ plus TE 50 mg/wk, and maximum DSG 300 $\mu\text{g/d}$ plus TE 100 mg/wk). These results suggest that DSG retains some androgenicity that, when combined with T, may be sufficient to induce the HDL reduction.

Discussion

In this paper we have reviewed published studies in which androgen-progestin hormonal combinations were administered to men for the purpose of sperm suppression. In the past 40 years (1960–2002) since the advent of female steroid contraception, 41 articles in which 802 male volunteers received an androgen-progestin combination have been published in peer-review journals.

Since the first progestins were administered to men, it has become clear that when administered alone progestins

Table 5. Rates of azoospermia with levonorgestrel and various testosterone formulations

	Testosterone (Oral)			Testosterone (Transdermal)		Testosterone Enanthate (IM)		Testosterone Undecanoate (IM)		Didrotestosterone (Transdermal)		References
	Dose	Azoo-spermia (%)		Dose	Azoo-spermia (%)	Dose	Azoo-spermia (%)	Dose	Azoo-spermia (%)	Dose	Azoo-spermia (%)	
Levonorgestrel												
Oral												
500 µg/d	200 mg/mo	0	Foegh et al, 1980
500 µg/d	200 mg/mo	40	Foegh et al, 1980
500 µg/d	100 mg/wk	67	Bebb et al, 1996
250 µg/d	200 mg/mo	20	Foegh et al, 1980
250 µg/d	100 mg/wk	78	Anawalt et al, 1999
250 µg/d	1000 mg/6 wk	50	Kamischke et al, 2000
250 µg/d	Foegh et al, 1980
250 µg/d × 12 wk then 500 µg/d	200-600 mg/d	0	...	18	Buchter et al, 1999
125 µg/d	100 mg/wk	61	Anawalt et al, 1999
30 µg/d	250 mg	0	Pollanen et al, 2001
125 µg/d	33	5 mg/d	Gonzalo IT, 2002
Implant												
150 mg n°2 implants	250 mg/mo	38	Geo E, 1999
100-30 µg n°1	250 mg	0	Pollanen et al, 2001
200-60 µg n°2	250 mg	0	Pollanen et al, 2001
400-120 µg n°4	250 mg	0	Pollanen et al, 2001
160 µg/d Norplant II (4 capsules)	35	5 mg/d × 2	Gonzalo, 2002
160 µg/d Norplant II (4 capsules)	100 mg/wk	93	Gonzalo, 2002

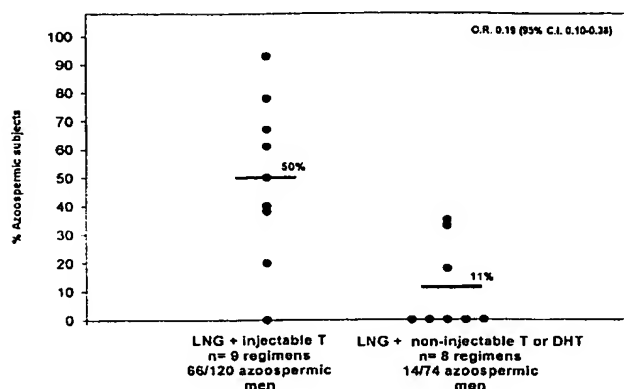


Figure 5. Rates of azoospermia after oral administration of levonorgestrel (LNG) in combination with injectable testosterone (T: testosterone enanthate [TE] or testosterone undecanoate [TU]) or noninjectable T (oral or transdermal). Full circles indicate rate of azoospermia achieved with each different regimen. Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing noninjectable vs injectable T regimens. See text and Table 5 for details.

induce symptoms of androgen deficiency due to gonadotropin suppression and possible direct effects on T metabolism. Even nor-progestins that are known to have a strong residual androgenic effect in women cannot maintain androgen-dependent physiological functions in men if given without androgens (Kamischke et al, 2000a). Therefore, for the purpose of male contraception all progestins available must be given in combination with androgens. The theoretical possibility of inducing profound gonadotropin suppression with the progestin alone would allow for the use of an androgen dose that is sufficient to maintain androgen-dependent physiological functions. However, available progestins have not been shown to induce such profound gonadotropin suppression when administered alone. Such a possibility remains open if new progestins are formulated that are more potent in terms of gonadotropin suppression at doses that do not cause adverse effects.

This analysis of the data published in the literature during these 4 decades suggests that when a progestin is administered in combination with an androgen, a profound suppression of spermatogenesis can be induced. Most studies suggest that the suppressive actions of progestin are additive to T. A wide range of degree of sperm suppression has been reported in the various studies with different doses and injection intervals. When optimal regimens were tested, all progestins induced azoospermia or near azoospermia in all men. Therefore, based on the data published so far, no progestin seems to be superior to the others in terms of spermatogenic suppression.

Throughout the different regimens, the low effectiveness of the androgen-progestin combinations that includ-

ed noninjectable androgens was consistently observed. Thirteen papers have reported on the combined administration of oral progestins with noninjectable androgens (Table 1a). Sperm suppression induced by these noninjectable androgen-progestin regimens is significantly lower compared with that achieved with regimens in which the 2 compounds have been given through injections or implants. The lower levels in serum T levels or wide fluctuations, as in the case of oral T preparations, can explain this low effectiveness. These lower and fluctuating levels may be unable to induce or maintain consistent gonadotropin suppression, as has been suggested by the comparison of gonadotropin levels between 2 groups in which CPA was given in combination with TE or oral TU (Meriggiola et al, 1996, 1997).

A few early studies proposed the idea of using a higher hormonal load to induce profound sperm suppression that can be eventually maintained with lower doses. In these preliminary trials, profound sperm suppression was induced with the initial high dosage. However, the steroid regimen administered to maintain suppression failed to do so (Alvarez-Sanchez et al, 1977, 1979; Frick et al, 1982). More recent studies in which different hormonal combinations have been used both in primates and in men have shown that it is indeed possible to maintain sperm suppression for as long as 32 weeks with lower hormone doses than those used to induce the suppression (Weinbauer et al, 1988; Swerdloff et al, 1998; Costantino et al, unpublished data). However, even these recent reports have given conflicting results (Behre et al, 2001). The use of inadequate regimens for maintenance of sperm suppression may account for the different ability to maintain gonadotropin, and thus sperm suppression, with the 2 regimens. However, this concept remains potentially very interesting and deserves to be explored further as the use of a lower hormonal load for long-term maintenance of sperm suppression may increase safety and decrease the cost, as well as increase acceptability and decrease side effects of the contraceptive.

None of these trials has reported major adverse effects that would discourage continuation of the hormonal combination. However, only years of clinical use will permit evaluation of the potential risks of hormone administration for contraception in men. It is speculated that avoidance of supraphysiological T levels results in a lower incidence of long-term adverse effects on health. When a progestin is added to the androgen for enhancement of sperm suppression, it must be administered at doses that are sometimes higher than those used in women. Therefore, at these dosages some activities of the progestin that are negligible in female contraceptives may reach physiological significance in male contraceptives. The choice of the progestin is also very important. The use of progestins with favorable biological and pharmacological

Table 6. Rates of azoospermia with desogestrel and various testosterone formulations

	Testosterone Enanthate (IM)		Testosterone Pellets (Subdermal)		Testosterone (Transdermal)		References
	Desogestrel	Dose	Azoospermia (%)	Dose	Azoospermia (%)	Dose	
Oral							
300 µg/d		100 mg/wk	75	Wu et al, 1999
300 µg/d		50 mg/wk	100	Wu et al, 1999
300 µg/d		100 mg/wk	88	Anawalt et al, 2000
150 µg/d		100 mg/wk	57	Wu et al, 1999
150 µg/d		50 mg/wk	57	Anawalt et al, 2000
150 µg/d		100 mg/wk	100	Anawalt et al, 2000
150 µg/d		400 mg	75	...	Kinniburgh et al, 2001
150 µg/d		400 mg + finasteride 5 mg/d oral	71	...	Kinniburgh et al, 2001
75 µg/d		5 mg/d	Morton Hair et al, 2001
150 µg/d		5 mg/d	Morton Hair et al, 2001
300 µg/d		5 mg/d	Morton Hair et al, 2001
300 µg/d		400 mg	100	...	Kinniburgh et al, 2002
150 µg/d		400 mg	71	...	Kinniburgh et al, 2002
Implant							
Etonogestrel (1 × 68 mg)		400 mg (2 × 200 mg)	64	...	Anderson et al, 2002
Etonogestrel (2 × 68 mg)		400 mg (2 × 200 mg)	75	...	Anderson et al, 2002

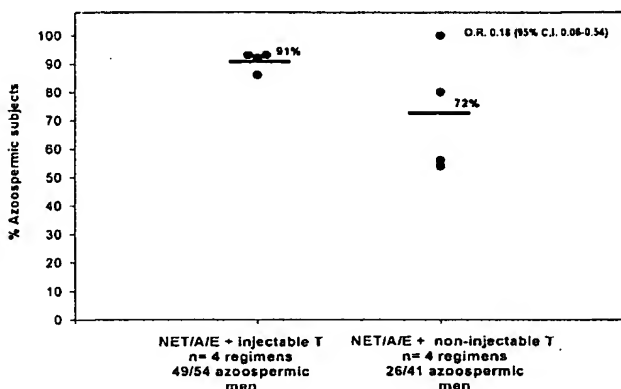


Figure 6. Rates of azoospermia after norethisterone (NET), NET enanthate (NETE), or NET acetate (NETA) and testosterone undecanoate (TU) injections or oral administration of NETA and noninjectable testosterone (T; oral or percutaneous). Full circles indicate rate of azoospermia achieved with each different regimen. Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing oral vs injectable T regimens. See text and Table 6 for details.

characteristics, which in this case means progestins devoid of strong androgenic or antiandrogenic activities at the doses used for induction of gonadotropin/sperm suppression, will certainly increase the long-term safety of these regimens.

Previous trials have shown that with the steroid formulations currently available, long-acting and depot formulations are more effective in inducing sperm suppression compared with the noninjectable formulations. Previous surveys reported that the majority of men from different countries agreed that injectable contraceptives would be more convenient compared with the currently available male contraceptives (Ringheim, 1999). Therefore, these long-acting androgen-progestin combinations promise to be welcomed by many men as a favorable alternative to currently available methods. A recent survey reported that men of different cultures find the pill more convenient to use compared with injections (Glasier, 1999). As with female contraception, the availability of a wide range of formulations of hormonal male contraceptives will allow for increased acceptability and compliance in men. Therefore, the task of developing noninjectable formulations that can be delivered orally or transdermally will remain a high priority in the male contraceptive agenda.

Conclusions

After so many years since the introduction of the female hormonal contraception, results of the latest studies have demonstrated that male hormonal contraception can become a reality. A recent cross-cultural survey has indicated that the majority of men would be willing to use

Table 7. Rates of azoospermia with norethisterone enanthate/acetate and various testosterone formulations

	Testosterone Undecanoate (Oral)		Testosterone (Percutaneous)		Testosterone Undecanoate (IM)		References
	Dose	Azoospermia (%)	Dose	Azoospermia (%)	Dose	Azoospermia (%)	
Norethisterone							
Enanthate (IM)							
200 mg/6 wk	1000 mg/6 wk	93	Kamischke et al, 2000
400 mg/6 wk	1000 mg/6 wk	92	Kamischke et al, 2002
200 mg/6 wk	1000 mg/6 wk	93	Kamischke et al, 2002
Acetate (Oral)							
5 mg/d	250 mg/d	80	Guerin et al, 1988
10 mg/d	250 mg/d	100	Guerin et al, 1988
10 mg/d	160 mg/d	54	Guerin et al, 1988
10 mg/d	1000 mg/6 wk	86	Kamischke et al, 2002
Net (Oral)							
5 mg/d	100 mg/d	56	Lobel et al, 1989

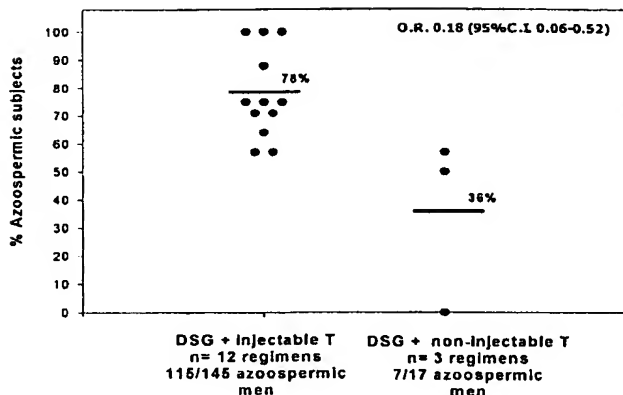


Figure 7. Rates of azoospermia after oral administration of desogestrel (DSG) in combination with injectable testosterone (T: testosterone enanthate [TE] or T pellets) or noninjectable T (transdermal). Full circles indicate rate of azoospermia achieved with each different regimen. Lines represent mean of the azoospermic rates in the 2 groups of studies. Odds ratio of azoospermia was calculated comparing noninjectable vs injectable T regimens. See text and Table 7 for details.

hormonal contraceptives and that women would trust their male partners to use them. Long-acting or depot injectable androgen-progestin formulations provide optimal sperm suppression with minimal short-term metabolic adverse effects that promise to result in high long-term safety of these regimens. Phase III multicenter clinical trials are now awaited for testing the contraceptive effectiveness of these hormonal regimens. Additionally, more research is needed to develop new steroid preparations with better biological properties, such as progestins with more potent gonadotropin activity, selective progestin modulators or long-acting androgen preparations, or new oral androgen formulations, to improve the long-term safety of these regimens and increase choice.

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Comparison of Spironolactone, Flutamide, and Finasteride Efficacy in the Treatment of Hirsutism: A Randomized, Double Blind, Placebo-Controlled Trial*

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ABSTRACT

To compare objectively the efficacies of spironolactone (100 mg/day), flutamide (250 mg/day), and finasteride (5 mg/day) in the treatment of hirsutism, 40 hirsute women were randomly assigned to double blind treatments with 1 of these 3 drugs or placebo for 6 months. Before and at the end of treatment, hirsutism was quantitatively measured in each subject by determination, by computer-assisted light microscopy, of the largest diameter of 5 hairs plucked from the linea alba. These measurements were averaged to produce a mean hair shaft diameter. For each subject, baseline and posttreatment assessments were carried out at the same time by an investigator blinded to both time and type of therapy. In addition, a semi-quantitative clinical evaluation was carried out by a modification of the Ferriman-Gallwey (F-G) scoring method, performed by a single investigator. At baseline the 4 groups of women had similar hair

diameters and F-G scores. After 6 months of therapy all groups of subjects given active drugs showed reductions of their hair diameters, without statistically significant differences among groups (mean change \pm SEM, $-11.7 \pm 5.6\%$, $-18.0 \pm 6.1\%$, and $-12.6 \pm 6.7\%$, respectively, in the spironolactone, flutamide, and finasteride groups). F-G scores were also significantly reduced in women receiving antiandrogen drugs, again without differences among groups (mean change, $-41.0 \pm 5.5\%$, $-38.9 \pm 7.2\%$, and $-31.6 \pm 3.7\%$, respectively). No significant changes from baseline values were recorded by either hair diameter ($-1.4 \pm 5.2\%$) or F-G score ($+5.4 \pm 3.7\%$) assessment in the placebo group. In conclusion, spironolactone, flutamide, and finasteride are all effective in the treatment of hirsutism. After a 6-month course of therapy, the clinical efficacies of these drugs, at least at the doses used, are similar. (*J Clin Endocrinol Metab* 85: 89–94, 2000)

HIRSUTISM is a very common clinical problem in endocrinological practice, with potentially serious psychosocial consequences (1). Mechanical hair removal is effective in many hirsute women. Nevertheless, a pharmacological approach is often required in subjects with moderate to severe hirsutism to suppress androgen production and/or action. Contraceptives are widely used for this purpose, but their efficacy is limited in established hirsutism (2). Although GnRH analogs may be indicated in selected patients (3), the most reliable therapeutic tool in these women is the use of antiandrogen drugs, usually in association with nonandrogenic oral contraceptive therapy (2).

Spironolactone and cyproterone acetate are the antiandrogens most commonly used in the treatment of hirsutism. Both of these steroidal compounds possess intrinsic hormonal activity and interfere with steroidogenesis (4, 5). In addition, cyproterone acetate shows significant antigonadotropic effects. Side-effects of these drugs include frequent menstrual

irregularity. The progestinic activity of cyproterone acetate requires this drug to be associated with estrogens.

Other antiandrogen drugs, such as flutamide and finasteride, have been proposed in the treatment of hirsutism. Flutamide is a nonsteroidal compound that seems to act only at the androgen receptor site and is therefore considered a pure antiandrogen (6). However, some data suggest that flutamide might also reduce the synthesis of androgens (7) and/or increase their metabolism to inactive molecules (8). This drug is efficaciously used in the treatment of advanced prostatic carcinoma and was successfully evaluated in hirsute women (9–11). Liver toxicity is a rare but potentially severe side-effect of flutamide (12).

Finasteride is a very potent competitive inhibitor of the type 2 isoenzyme of 5 α -reductase, the enzyme responsible for conversion of testosterone to the active metabolite dihydrotestosterone (13). The drug has recently been approved for the treatment of benign prostatic hyperplasia (14). As increased 5 α -reductase activity is considered a pathogenetic mechanism of hirsutism (15), selective enzyme inhibition has been proposed as a rational medical approach to this condition as well. Consistently, studies in hirsute women gave promising results, without appreciable side-effects (16–18).

Rigorous clinical trial methodology is very rarely encountered in reports evaluating antiandrogenic therapies in hirsute women (1, 19, 20). Furthermore, the majority of researchers who previously assessed the effects of these drugs

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used subjective parameters, namely the Ferriman-Gallwey (F-G) (21) or other similar scoring systems, as the only methods to measure hirsutism, making comparison of data among different studies unreliable (19). In addition, in several studies antiandrogens were given in combination with oral contraceptives, making it difficult to quantify the therapeutic efficacies of the antiandrogen compound and the estrogens separately. Until now only a few studies directly compared the clinical efficacies of different antiandrogen drugs in hirsute women. Although some of these studies used objective methods to measure hair growth (17, 22, 23), none of them was double blind or placebo controlled. Thus, the scale of relative potency of these drugs remains undetermined.

To address this issue, the present study compared the clinical efficacy, in 40 hirsute women, of a 6-month course of double blind, placebo-controlled treatments with spironolactone, flutamide, or finasteride. Hair growth was estimated by both a modification of the F-G score and the objective measurement of shaft diameters of hairs plucked from the linea alba.

Materials and Methods

Subjects

Forty young women (age, 20.4 ± 0.5 yr; body mass index, 24.5 ± 0.7 kg/m²; mean \pm SEM), consecutively referred to our division for moderate to severe hirsutism, were included in the study. The mean modified F-G score (24) was 17.5 ± 0.7 (range, 11–27.5). Two subjects also suffered from mild acne.

Twenty-one women had polycystic ovary syndrome, diagnosed according to the presence of hyperandrogenism and chronic anovulation (25). All of them showed a 17-hydroxyprogesterone hyperresponse to GnRH agonist testing (26). Eighteen of these subjects had oligomenorrhea.

Cushing's syndrome, adrenal enzyme defects, adrenal and ovarian tumors, hyperprolactinemia, and thyroid dysfunction were excluded in each subject. No patient suffered from any other disease or had been treated with oral contraceptives or antiandrogen drugs in the previous 12 months.

Patients were clearly informed of potential risks of the treatments, with a particular caution to avoid pregnancy because of possible male fetus feminization. Sexually active women were advised to use barrier contraceptive methods or intrauterine devices during the study. The study was conducted in accordance with the Declaration of Helsinki on human experimentation. Each patient gave her written informed consent to the study protocol, which was approved by the local ethical committee.

Protocol

Patients were randomly assigned to double blind treatments, once daily orally as a wafer capsule, for 6 months with one of the following: 1) spironolactone (100 mg), 2) flutamide (250 mg), 3) finasteride (5 mg), or 4) placebo. Basally and at the end of treatment, hirsutism and hormonal parameters were evaluated in each subject, as described below.

Patients were instructed to report any untoward effect during the treatment period. In addition, safety parameters were assessed before treatment and at 2-month intervals during the study. The safety evaluation comprised hematology tests (hemoglobin, red blood cells, white blood cells, platelets, and leukocyte differential count) and biochemistry tests (plasma glucose, uric acid, liver and renal function, and serum electrolytes). To be considered clinically significant, changes in safety parameters had to either exceed the normal limits or double the baseline values.

Hirsutism assessment

Hair growth assessment included both an objective method and a hirsutism score, graded by a modified F-G method. An objective eval-

uation of hirsutism in each patient was obtained by averaging the largest diameters of five hairs plucked, using regular facial tweezers, from a 2×2 -cm area in the linea alba. To avoid any hair damage, hairs were not cut, shaved, or submitted to any cosmetic procedures in this specific area for 3 months beforehand. Plucking was immediately followed by shaving, and no additional cosmetic measure was allowed in this body region throughout the study. Hairs were gently embedded in Eukitt (mounting medium O, Kindler GmbH & Co., Freiburg, Germany) on a glass microslide, using a small anatomical forceps; they were mounted longitudinally on the slide and protected by a cover glass previously bathed with xilol.

The slides were examined using a fully integrated, optical microscope (Leitz DMRB, Wetzlar, Germany), with a 12-V, 100-watt halogen illuminator for transmitted light and a computer workstation system, the CAS 200/486 imaging analysis system (Cell Analysis Systems, Inc., Elmhurst, IL). This device included an IBM enhanced AT computer, two full-color display monitors, a graphics and system control/menu selection monitor, and a digital image display for displaying real-time digital images. Internally, images are stored at 256×256 pixel resolution, with the corresponding image resolution determined by the objective lens chosen (27). The image analysis program used was Micrometer version 0.7, application 1992 (Cell Analysis Systems, Inc., version 1.0). Hair shaft diameter was measured at $\times 10$ magnification, and the Video Trace mode was used for drawing a line from one point to another. Vertical resolution was $0.0766053/\mu\text{m}$, and horizontal resolution was $0.0446314/\mu\text{m}$. The hair shaft diameter was measured just above the keratogenous zone. This zone appears as a dark area of the hair shaft located above the hair bulb. In this area, hair matrix cells undergo complete keratinization, and the hair fiber decreases in diameter by about 25%, mainly because of water loss. A mean hair shaft diameter was obtained for each patient by averaging the measurements obtained from the anagen hairs present in the sample.

All measurements relative to each patient were made at the same time by a single investigator blinded to both time and type of therapy. With this method, both intra- and intersubject mean coefficients of variation were less than 2%.

A semiquantitative, clinical evaluation of hirsutism was also performed in these subjects by a modification of the F-G method (24). The hirsutism scores were determined twice in the pretreatment period, with a 3-month interval between measurements, and subsequently at the end of the 6-month treatment period. Patients using cosmetic measures were requested not to depilate for at least 1 month before each evaluation. All evaluations were carried out by a single investigator, blinded to the ongoing therapy. The mean difference in hirsutism scores for each subject between the two baseline evaluations was 0.4 (range, 0–3.5).

The patients' subjective opinion of the clinical outcome of therapy (excellent, good, fair, or poor) was also obtained. In addition, before and at the end of treatment each woman completed a questionnaire specifying any cosmetic measures for hair removal, with details of type (plucking, waxing, shaving, bleaching, or depilatory cream), site, and frequency.

Endocrine assessments

A standard hormonal profile, including serum gonadotropins and androgens (total and free testosterone, dehydroepiandrosterone sulfate, androstenedione, and 3α -androstenediol glucuronide), was determined at baseline and at the end of treatment from a blood sample obtained at 0800 h. Twenty-four-hour urine was collected at the same time points for C₁₉ and C₂₁ steroid metabolite determinations. In the eumenorrheic patients blood samples were collected in the early follicular phase of the menstrual cycle, whereas in the oligomenorrheic subjects luteal phase was excluded by serum progesterone assay.

Assays

Serum hormones were measured by commercial kits, as previously described (16). All samples from each patient were run in the same assay, in duplicate. Urinary steroid metabolites were assayed by gas chromatography, as previously described (16).

Statistics and calculations

Results were analyzed by Student's *t* test for paired and unpaired data, Wilcoxon's rank sum test, and ANOVA. All tests of significance were two tailed, and $P \leq 0.05$ was considered significant. Etiocholanolone/androsterone and tetrahydrocortisol/allotetrahydrocortisol urinary metabolite ratios were calculated as indexes of 5 α -reductase activity. Data were expressed as the mean \pm SEM.

Results

Tolerability

Metrorrhagias were reported by five women, all given spironolactone. This side-effect was transient in four subjects and sustained in one, but not so severe as to require interruption of treatment. On the other hand, previous menstrual abnormalities improved after treatment in five patients (three in the flutamide group and one in each of the other groups receiving active drugs).

One patient receiving flutamide complained of sleepiness and hyporexia, which spontaneously disappeared after the first month of therapy. One woman in the finasteride group complained of a transient sensation of being "swollen." Finally, one patient in the placebo group reported mild, transient headache and nausea.

Safety parameters did not show significant changes in any subject during the study period.

Hirsutism

At baseline, mean shaft diameters of hairs plucked from the linea alba and modified F-G scores were comparable in the four groups of hirsute women (Table 1). After 6 months of therapy, all groups of subjects given active drugs showed significant decreases in hair diameter (Fig. 1), without differences among groups (144 ± 12 vs. 164 ± 8 , 139 ± 10 vs. 172 ± 13 , and 146 ± 8 vs. 172 ± 9 μ m, respectively, in the spironolactone, flutamide, and finasteride groups; all $P < 0.01$ vs. changes in the placebo group). Hirsutism scores also showed significant improvements in women receiving antiandrogen drugs (Fig. 1), without statistically significant differences among groups (10.0 ± 1.1 vs. 16.9 ± 0.9 , 11.1 ± 1.8 vs. 17.5 ± 1.5 , and 13.0 ± 1.3 vs. 18.4 ± 1.3 in the three groups; all $P < 0.001$ vs. placebo). Similar improvements were found in women with polycystic ovary syndrome and in those with nonovarian hyperandrogenism considered as a whole, regardless of treatment group (changes in hair diameter, $-11.9 \pm 4.8\%$ vs. $-15.7 \pm 5.2\%$; changes in hirsutism

TABLE 1. Baseline clinical characteristics of hirsute women divided according to treatment

	Spironolactone	Flutamide	Finasteride	Placebo	<i>P</i> ^a
No.	10	10	10	10	
Age (yr)	19.7 ± 0.7	20.2 ± 1.1	19.8 ± 0.8	21.8 ± 1.3	NS
BMI (kg/m ²)	25.3 ± 1.4	23.6 ± 1.0	23.3 ± 0.7	25.8 ± 2.0	NS
Modified Ferriman-Gallwey score	16.9 ± 0.9	17.5 ± 1.5	18.4 ± 1.3	17.2 ± 1.6	NS
Hair diameter (μ m)	164 ± 8	172 ± 13	172 ± 9	153 ± 7	NS
Menses (irregular/regular)	2/8	8/2	4/6	4/6	NS
PCOS/other	4/6	8/2	4/6	5/5	NS
Cosmetic measures (yes/no)	5/5	4/6	8/2	7/3	NS

^a By ANOVA or χ^2 , as appropriate.

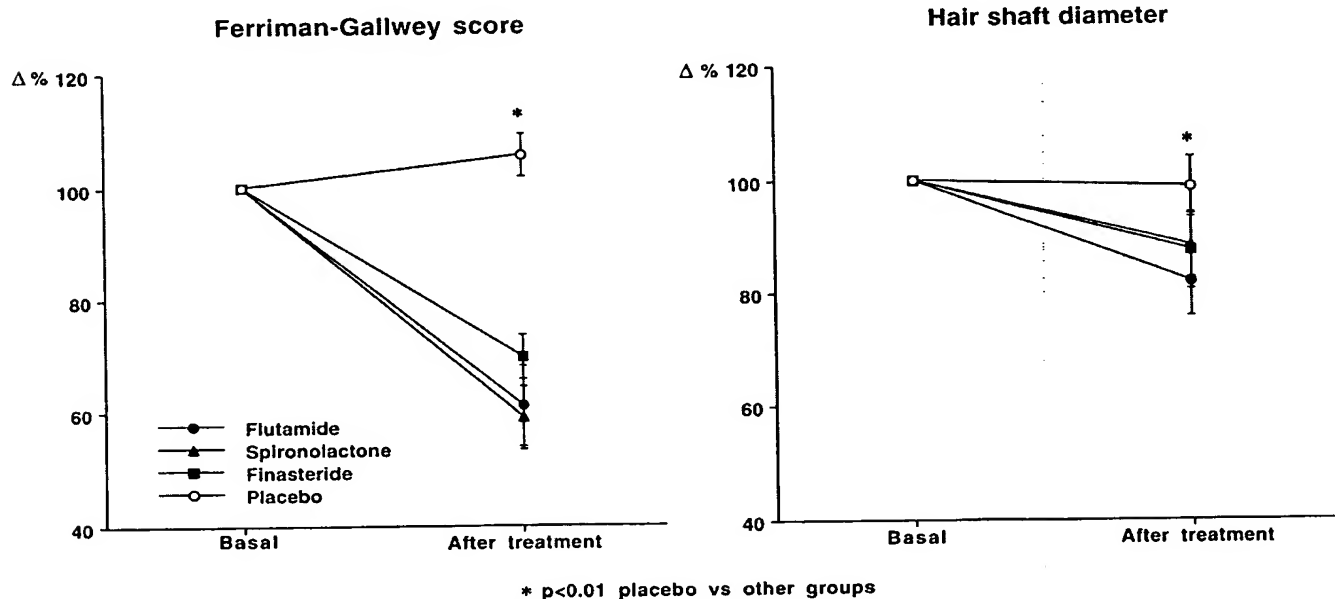


FIG. 1. Changes after therapy ($\Delta\%$) in modified F-G score and mean hair shaft diameter in the four treatment groups.

score, $-41.6 \pm 4.3\%$ vs. $-31.0 \pm 4.8\%$). No significant changes from baseline values were found by either hair diameter (156 ± 11 vs. $153 \pm 7 \mu\text{m}$) or hirsutism score (18.0 ± 1.7 vs. 17.2 ± 1.6) in the placebo group.

Before treatment 24 women were using cosmetic measures. These consisted of waxing ($n = 20$), waxing and plucking ($n = 2$), waxing and shaving ($n = 1$), or depilatory cream ($n = 1$), with variable frequency (2–30 days). Changes in these procedures were reported only by patients given active drugs. Four of these subjects (2 in the spironolactone and 2 in the flutamide groups) stopped waxing. All of the other 13 women given antiandrogens reported a reduction in frequencies of hair removal procedures, without substantial differences among groups (data not shown). On the other hand, no change was reported by women receiving placebo.

Patients' self-evaluations of clinical outcome at the end of the study were also consistent with changes in hair diameter and hirsutism score (Table 2).

Clinical and endocrine features

Tables 1 and 3 show the main clinical and endocrine features of women in the four groups, before and during the trial. At baseline all groups showed similar characteristics. The frequency of menstrual irregularities was higher in the flutamide group, but this difference was not statistically significant.

After treatment a significant reduction of serum dehydroepiandrosterone sulfate was found in the flutamide group, whereas 3α -androstenediol glucuronide levels, an index of 5α -reductase activity, were significantly reduced in the finasteride group. Consistently, women given finasteride also showed significant increases in C_{19} and C_{21} urinary $5\beta/5\alpha$ -steroid metabolite ratios (data not shown). Free testosterone showed a tendency to decrease in the flutamide group and to increase in the finasteride group, but these differences did not reach statistical significance. Women given spironolac-

tone as well as those given placebo did not show any changes in endocrine parameters.

Discussion

The vast majority of the published work concerning hirsutism treatment shows considerable shortcomings, such as lack of a control group, assessment of results only by subjective methods, or too short a duration in relation to the physiology of hair growth (1, 19, 20). These limitations make it at present impossible to establish a scale of relative potency among antiandrogen drugs. Indeed, some of the few controlled studies were even unable to demonstrate differences in hair growth during antiandrogen therapy vs. placebo administration (4). Furthermore, until now only a limited number of studies directly compared the clinical efficacies of different antiandrogen drugs in the treatment of hirsute women (17, 22, 23, 28–34). Although some of these studies were randomized (17, 22, 28, 31), only a few used objective measures of hair growth (17, 22, 23), and none of them was double blind or placebo controlled.

The present study is the first to evaluate the efficacy on hirsutism of three different drugs, spironolactone, flutamide, and finasteride, by a rigorous clinical methodology of a double blind, placebo-controlled, randomized study. Furthermore, hair growth changes were measured not only by the subjective F-G scoring, commonly used in clinical practice as a semiquantitative measure of degree of hirsutism, but also by an objective method, measurement of hair shaft diameters. This method was previously validated and used in clinical trials (19), although its sensitivity, at least using simple optical microscopy, is not high (3, 19). To increase both the sensitivity and reproducibility of this procedure, in the present study a computer-assisted measurement was performed. On the other hand, although other procedures have been proposed for the objective measurement of hair growth, at present none of them may be considered a gold standard (19). Our data were further supported by consistent results of patients' self-evaluation of clinical outcome and by changes in frequency of recourse to mechanical hair removal.

In this study the changes in F-G score were 2–3 times greater than those in hair diameter. This observation is not surprising, as subjective perception of hair growth excess

TABLE 2. Patients' self-evaluation of clinical outcome of the therapy

	Spironolactone	Flutamide	Finasteride	Placebo
Excellent	3	4	1	0
Good	1	4	4	0
Fair	5	1	4	1
Poor	1	1	1	9

TABLE 3. Standard hormonal profiles, before and after treatment, in the four treatment groups

	Spironolactone		Flutamide		Finasteride		Placebo	
	Basal	After treatment	Basal	After treatment	Basal	After treatment	Basal	After treatment
Free testosterone (pg/mL)	3.47 ± 0.31	3.43 ± 0.24	3.36 ± 0.45	2.78 ± 0.27	3.50 ± 0.41	4.24 ± 0.42	3.21 ± 0.29	3.25 ± 0.32
DHEA-S ($\mu\text{g/L}$)	2177 ± 319	2336 ± 270	1962 ± 231	1349 ± 188^a	2380 ± 175	2079 ± 183	2158 ± 229	1707 ± 134
3α -Androstenediol glucuronide (ng/mL)	4.24 ± 0.56	5.64 ± 0.66	4.05 ± 0.71	3.54 ± 0.40	6.40 ± 1.23	2.86 ± 0.78^a	5.05 ± 0.80	5.06 ± 0.64
LH (IU/L)	7.7 ± 1.4	8.3 ± 1.4	5.5 ± 0.9	8.3 ± 1.7	6.5 ± 1.1	6.6 ± 1.5	7.4 ± 1.1	7.1 ± 1.7
FSH (IU/L)	4.9 ± 0.2	5.3 ± 0.2	5.1 ± 0.3	4.8 ± 0.4	4.9 ± 0.2	4.7 ± 0.5	5.5 ± 0.4	5.6 ± 0.5

Values are the mean \pm SEM.

^a $P < 0.01$ vs. basal.

depends not only on hair diameter, but also on the length and density of terminal hairs.

The present controlled trial demonstrates that spironolactone, flutamide, and finasteride are effective in the treatment of hirsutism, supporting conclusions of previous, almost all uncontrolled, studies (17, 22, 23, 28–30, 32). However, the most interesting finding of the present study is that, in a population of unselected women with moderate to severe hirsutism, the clinical efficacies of these drugs were similar despite their differing mechanisms of action.

Spironolactone has been given to hirsute women in previous studies in doses ranging from 50–400 mg/day (4). The large majority of researchers used daily doses of 100 mg, as in the present trial, because an increased frequency of side-effects is associated with higher doses (35). Flutamide, too, was previously given to hirsute women in a large range of doses, from 250–750 mg/day (10, 11, 36, 37). We chose the lowest of these amounts to minimize any potential risk of liver toxicity. Furthermore, a dose-range study reported similar improvements in hirsutism with 250 vs. 500 mg flutamide (37). On the other hand, 5 mg finasteride is the dose used in all published trials with this drug, although similar effects on skin androgens were found with 1 mg (38).

We cannot exclude that by using different doses of drugs or by extending the duration of treatments it might be possible to identify some differences in clinical efficacy among these drugs. Nevertheless, differences of clinical relevance are unlikely. This opinion is supported by the observation that the extent of improvements in F-G score in this study was roughly comparable with previously reported results at higher doses of these antiandrogens (4, 36, 37), although in a short term (3-month) trial Lobo *et al.* (39) showed greater reduction of hair shaft diameters with 200 than 100 mg/day spironolactone. Another potential bias is the different percentage of ovarian vs. nonovarian hyperandrogenism in our treatment groups, although this difference did not reach statistical significance. However, considering the whole population of women receiving active drugs, we did not find any difference in improvement of hirsutism between subjects with polycystic ovary syndrome and those with other forms of androgen excess.

The tolerability of antiandrogen drugs examined in the study was good, with the noticeable exception of polymenorrhea in 50% of subjects given spironolactone. This is a well known adverse effect of the drug. However, in most of these women the side-effect was transient, resolving within 3 months. No patient receiving flutamide showed liver toxicity. This side-effect of flutamide showed a low incidence (<0.5%), but was occasionally fatal in large populations of men with advanced prostate carcinoma treated with 750–1500 mg/day (12). We and others previously also observed occasional mild, transient hepatotoxicity in women given low doses of the drug (375–500 mg/day) (11, 40). As a whole, these observations suggest that the use of this compound for the treatment of hirsutism should be carefully challenged in each subject. On the other hand, Diamanti-Karamandis *et al.* recently reported that flutamide has favorable effects on lipid profile in women with polycystic ovarian syndrome (41); this effect is of clinical interest in subjects who frequently show several metabolic abnormalities (42). From the point of view

of tolerability, finasteride, devoid of appreciable side-effects, seems to be the current best choice. It should be borne in mind that all antiandrogens imply the need to avoid a pregnancy, given the potential risk of feminization of male fetuses.

Finally, at the doses used in this study the retail costs of a 1-month course of therapy are \$21.3, \$95.4, and \$56.1, respectively for spironolactone, flutamide, and finasteride (source of data: drugstore.com web site, July 26, 1999). This aspect should also be considered in the choice of an antiandrogen therapy, particularly as the efficacies of these drugs are similar.

In conclusion, this double blind, placebo-controlled study demonstrates that spironolactone, flutamide, and finasteride are all effective in the treatment of hirsutism. Moreover, after a 6-month course of therapy the clinical efficacies of these drugs are similar. Further research should investigate the potential for synergic effects of combined therapies with drugs acting at different levels in androgen secretion and/or action.

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The effect of cyproterone acetate and orchiectomy on metastases from prostatic cancer.

Pescatore D, Giberti C, Martorana G, Natta G, Giuliani L.

We have studied 38 patients with previously untreated, widespread prostatic cancer, who were submitted to therapy with cyproterone acetate and orchiectomy. 70% of patients with symptoms have shown subjective improvement. Moreover, it was possible to observe the regression, stabilization or progression of metastases in 32, 50 and 18% of the cases, respectively. We conclude that such therapy is effective in the initial treatment of metastatic prostate cancer and can be continued under radiographic control until the appearance of new metastases. At this point radiotherapy and alkylating agents may be effective.

PMID: 7371660 [PubMed - indexed for MEDLINE]

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Anticholinergic: Dry mouth, blurred vision, disturbance of accommodation, constipation, paralytic ileus, urinary retention, dilatation of urinary tract.

Cardiovascular: Hypertension, hypertension, tachycardia, arrhythmias, myocardial infarction, arrhythmias, heart failure.

Neuromuscular: Confusional states, disturbed orientation, disorientation, delusions, hallucinations, jitteriness, anxiety, restlessness, insomnia, tremors, numbness, tingling, and paresthesias of the extremities, peripheral neuropathy, incoordination, ataxia, seizures, alteration in EEG patterns, extrapyramidal symptoms, tinnitus.

Testicular: swelling and gynecomastia in the male, breast enlargement and galactorrhea in the female, decreased libido, elevation and lowering of blood levels, syndrome of inappropriate ADH (antidiuretic hormone secretion).

Gastrointestinal: Nausea, epigastric distress, heartburn, anorexia, stomatitis, peculiar taste, diarrhea, parotid swelling, black tongue. Rarely, hepatitis occurred (including altered liver function and jaundice).

Hematologic: Bone marrow depression including agranulocytosis, leukopenia, eosinophilia, purpura, thrombocytopenia.

Other: Dizziness, weakness, fatigue, headache, weight gain, increased perspiration, urinary frequency, mydriasis, alopecia.

Withdrawal Symptoms: Abrupt cessation of treatment or prolonged administration may produce nausea, headache, and malaise. These are not indicative of addiction.

DOSE AND ADMINISTRATION

Initial Dosage
Psychoneurotic patients whose anxiety and depression are combined therapy, one ETRAFON Tablet (2-25) or ETRAFON-FORTE Tablet (4-25) three or four times a day as recommended.

Maintenance Dosage
Elderly patients, adolescents and other patients as indicated, one ETRAFON-A Tablet (4-10) may be administered two or four times a day as initial dosage. This dosage may be adjusted as required to produce an adequate response.

Severe Illness
In more severely ill patients with schizophrenia, two ETRAFON-FORTE Tablets (4-25) three times a day are recommended as the initial dosage. If necessary, a fourth may be given at bedtime. The total daily dosage should not exceed eight tablets of any strength.

Maintenance Dosage
Depending on the condition being treated, the onset of therapeutic response may vary from a few days to a few weeks or longer. After a satisfactory response is noted, dosage should be reduced to the smallest dose which is effective for relief of the symptoms for which ETRAFON Tablets are being administered. A useful maintenance dosage is one ETRAFON Tablet (2-25) or one ETRAFON-FORTE Tablet (4-25) two to four times a day. In some patients, maintenance dosage is required for many months.

ETRAFON-A 2-10 Tablets (2-10) and ETRAFON-A 4-10 Tablets (4-10) can be used to increase flexibility in adjusting maintenance dosage to the lowest amount consistent with relief of symptoms.

ADVERSE REACTIONS

In the event of overdose, emergency treatment should be initiated immediately. All patients suspected of having taken an overdose should be hospitalized as soon as possible.

Manifestations: Overdosage of perphenazine primarily involves the extrapyramidal mechanism and produces the same side effects described under ADVERSE REACTIONS, but to a more marked degree. It is usually evidenced by stupor, coma, children may have convulsive seizures.

High doses of ETRAFON Tablets may cause temporary confusion, disturbed concentration, or transient visual hallucinations. Overdosage may cause drowsiness, hypothermia, tachycardia and other arrhythmic abnormalities—for example, bundle branch block; ECG evidence of impaired conduction; congestive heart failure; dilated pupils; convulsions; severe hypotension; stupor; and coma. Other symptoms may be agitation, hyperactive reflexes, muscle rigidity, vomiting, hyperpyrexia, or any of the adverse reactions listed for perphenazine or amitriptyline hydrochloride.

Overdosage with tricyclic antidepressants (TCAs), such as amitriptyline, doxepin, or amitriptyline may result in plasma TCA levels of 1,000 ng/ml or higher. Such levels more accurately define patients who are at risk for major medical complications of overdose than does the amount of drug ingested based on patient history. In one study, all patients with plasma TCA levels of this magnitude had a QRS duration of 100 msec or more on a routine ECG within the first 24 hours following overdose.

The absence of TCA blood level determinations, a QRS of 100 msec or more suggests a greater likelihood of serious complications.

Motor paresis (loss of conjugate movement in the so-called doll's eyes maneuver) as a manifestation of amitripty-

line overdosage has been reported as being significant in the differential diagnosis of a patient in light coma.

Treatment: Treatment is symptomatic and supportive. There is no specific antidote. The patient should be induced to vomit even if emesis has occurred spontaneously. Pharmacologic vomiting by the administration of ipecac syrup is a preferred method. It should be noted that ipecac has a central mode of action in addition to its local gastric irritant properties, and the central mode of action may be blocked by the antiemetic effect of ETRAFON Tablets. Vomiting should not be induced in patients with impaired consciousness. The action of ipecac is facilitated by physical activity and by the administration of 8 to 12 fluid ounces of water. If emesis does not occur within 15 minutes, the dose of ipecac should be repeated. Precautions against aspiration must be taken, especially in infants and children. Following emesis, any drug remaining in the stomach may be adsorbed by activated charcoal administered as a slurry with water. If vomiting is unsuccessful or contraindicated, gastric lavage should be performed. Isotonic and one-half isotonic saline are the lavage solutions of choice. Saline cathartics, such as milk of magnesia, draw water into the bowel by osmosis and therefore may be valuable for their action in rapid dilution of bowel content.

Standard measures (oxygen, intravenous fluids, corticosteroids) should be used to manage circulatory shock or metabolic acidosis. An open airway and adequate fluid intake should be maintained. Body temperature should be regulated. Hypothermia is expected, but severe hyperthermia may occur and must be treated vigorously. (See CONTRAINDICATIONS.)

An electrocardiogram should be taken and close monitoring of cardiac function instituted if there is any sign of abnormality. Cardiac arrhythmias may be treated with neostigmine, pyridostigmine, or propranolol. Digitalis should be considered for cardiac failure. Close monitoring of cardiac function is advisable for not less than five days.

Vasopressors such as norepinephrine may be used to treat hypotension, but epinephrine should NOT be used.

The intravenous administration of 1 to 3 mg physostigmine salicylate has been reported to reverse the symptoms of tricyclic antidepressant poisoning and therefore, should be considered in the symptomatic treatment of the central anticholinergic effects due to overdosage with ETRAFON Tablets. Because physostigmine is rapidly metabolized, it should be re-administered as required, especially if life-threatening signs, such as arrhythmias, convulsions, or deep coma recur or persist.

Anticonvulsants (an inhalation anesthetic, diazepam, or paraldehyde) are recommended for control of convulsions; since perphenazine increases the central nervous system depressant action, but not the anticonvulsant action of barbiturates.

If acute parkinson-like symptoms result from perphenazine intoxication, benztropine mesylate or diphenhydramine may be administered.

Central nervous system depression may be treated with non-convulsant doses of CNS stimulants. Avoid stimulants that may cause convulsions (e.g., picrotoxin and pentylenetetrazol).

Signs of arousal may not occur for 48 hours.

Dialysis is of no value because of low plasma concentrations of the drug.

Since overdosage is often deliberate, patients may attempt suicide by other means during the recovery phase. Deaths by deliberate or accidental overdosage have occurred with this class of drugs.

HOW SUPPLIED

ETRAFON 2-10 Tablets (perphenazine 2 mg and amitriptyline hydrochloride 10 mg): deep yellow, sugar-coated tablets branded in blue-black with the Schering trademark and either product identification letters, ANA, or number, 287; bottles of 100 (NDC 0085-0287-04) and 500 (NDC 0085-0287-07) and box of 100 for unit-dose dispensing (10 strips of 10 tablets each) (NDC 0085-0287-08).

ETRAFON Tablets (perphenazine 2 mg and amitriptyline hydrochloride 25 mg): pink, sugar-coated tablets branded in red with the Schering trademark and either product identification letters, ANC or number, 598; bottles of 100 (NDC 0085-0598-04) and 500 (NDC 0085-0598-07) and box of 100 for unit-dose dispensing (10 strips of 10 tablets each) (NDC 0085-0598-08).

ETRAFON-A Tablets (perphenazine 4 mg and amitriptyline hydrochloride 10 mg): orange, sugar-coated tablets branded in blue-black with the Schering trademark and either product identification letters, ANB, or number, 119; bottles of 100 (NDC 0085-0119-04) and box of 100 for unit-dose dispensing (10 strips of 10 tablets each) (NDC 0085-0119-08).

ETRAFON-FORTE Tablets (perphenazine 4 mg and amitriptyline hydrochloride, 25 mg): red, sugar-coated tablets branded in blue with the Schering trademark and either product identification letters, ANE, or number, 720; bottles of 100 (NDC 0085-0720-04) and 500 (NDC 0085-0720-07) and box of 100 for unit-dose dispensing (10 strips of 10 tablets each) (NDC 0085-0720-08).

Store ETRAFON 2-10, 4-10, 2-25 and 4-25 Tablets between 2° and 25°C (36° and 77°F). In addition, protect unit-dose packages from excessive moisture.

Revised 6/87

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Shown in Product Identification Section, page 427

EULEXIN®

brand of flutamide
Capsules

DESCRIPTION

EULEXIN Capsules contain flutamide, an acetanilid, non-steroidal, orally active antiandrogen having the chemical name, 2-methyl-N[4-nitro-3-(trifluoromethyl)phenyl]propanamide.

Each capsule contains 125 mg flutamide. The compound is a buff to yellow powder with a molecular weight of 276.2. The inactive ingredients for EULEXIN Capsules include: corn starch, lactose, magnesium stearate, povidone, and sodium lauryl sulfate. Gelatin capsule shells may contain methylparaben, propylparaben, butylparaben and the following dye systems: FD&C Blue 1, FD&C Yellow 6 and either FD&C Red 3 or FD&C Red 40 plus D&C Yellow 10, with titanium dioxide and other inactive ingredients.

CLINICAL PHARMACOLOGY

General: In animal studies, flutamide demonstrates potent antiandrogenic effects. It exerts its antiandrogenic action by inhibiting androgen uptake and/or by inhibiting nuclear binding of androgen in target tissues or both. Prostatic carcinoma is known to be androgen-sensitive and responds to treatment that counteracts the effect of androgen and/or removes the source of androgen, e.g., castration.

Pharmacokinetics: Analysis of plasma, urine, and feces following a single oral 200 mg dose of tritium-labeled flutamide to human volunteers showed that the drug is rapidly and completely absorbed. It is excreted mainly in the urine with only 4.2% of the dose excreted in the feces over 72 hours. The composition of plasma radioactivity showed that flutamide is rapidly and extensively metabolized, with flutamide comprising only 2.5% of plasma radioactivity one hour after administration. At least six metabolites have been identified in plasma. The major plasma metabolite is a biologically active, alpha-hydroxylated derivative which accounts for 23% of the plasma tritium one hour after drug administration.

The major urinary metabolite is 2-amino-5-nitro-4-(trifluoromethyl)phenol.

Following a single 250 mg oral dose to normal adult volunteers, low plasma levels of varying amounts of flutamide were detected. The biologically active alpha-hydroxylated metabolite reaches maximum plasma levels in about two hours, indicating that it is rapidly formed from flutamide. The plasma half-life for this metabolite is about 6 hours. Following multiple oral dosing of 250 mg t.i.d. in normal geriatric volunteers, flutamide and its active metabolite approached steady-state plasma levels (based on pharmacokinetic simulations) after the fourth flutamide dose. The half-life of the active metabolite in geriatric volunteers after a single flutamide dose is about 8 hours and at steady-state is 9.6 hours.

Flutamide, *in vivo*, at steady-state plasma concentrations of 24 to 78 ng/mL is 94% to 96% bound to plasma proteins. The active metabolite of flutamide, *in vivo*, at steady-state plasma concentrations of 1556 to 2284 ng/mL, is 92% to 94% bound to plasma proteins.

In male rats neither flutamide nor any of its metabolites is preferentially accumulated in any tissue except the prostate after an oral 5 mg/kg dose of ¹⁴C-flutamide. Total drug levels were highest 6 hours after drug administration in all tissues. Levels declined at roughly similar rates to low levels at 18 hours. The major metabolite was present at higher concentrations than flutamide in all tissues studied.

Elevations of plasma testosterone and estradiol levels have been noted following flutamide administration. Clinical Studies: Flutamide has been demonstrated to interfere with testosterone at the cellular level. This can complement medical castration achieved with leuprolide, which suppresses testicular androgen production by inhibiting luteinizing hormone secretion.

To study the effects of combination therapy, 617 patients (311 leuprolide + flutamide, 306 leuprolide + placebo) with previously untreated advanced prostatic carcinoma were enrolled in a large multi-centered, controlled clinical trial. Three and one-half years after the study was initiated, median survival had been reached. The median actuarial survival time was 34.9 months for patients treated with leuprolide.

Continued on next page

Information on Schering products appearing on these pages is effective as of August 15, 1991.

Schering—Cont.

lide and flutamide versus 27.9 months for patients treated with leuprolide alone. This seven month increment represents 25% improvement in overall survival with the flutamide therapy. Analysis of progression free survival showed a 2.6 month improvement in patients who received leuprolide plus flutamide, a 19% increment over leuprolide and placebo.

INDICATIONS AND USAGE

EULEXIN Capsules are indicated for use in combination with LHRH agonistic analogues (such as leuprolide acetate) for the treatment of metastatic prostatic carcinoma (stage D₂). To achieve the benefit of the adjunctive therapy with EULEXIN, treatment must be started simultaneously using both drugs.

CONTRAINDICATIONS

EULEXIN Capsules are contraindicated in patients who are hypersensitive to flutamide or any component of this preparation.

WARNINGS

Gynecomastia occurred in 9% of patients receiving flutamide together with medical castration.

Flutamide may cause fetal harm when administered to a pregnant woman. There was decreased 24-hour survival in the offspring of rats treated with flutamide at doses of 30, 100, or 200 mg/kg/day (approximately 3, 9, and 19 times the human dose) during pregnancy. A slight increase in minor variations in the development of the sternbrae and vertebrae was seen in fetuses of rats at the two higher doses. Feminization of the males also occurred at the two higher dose levels. There was a decreased survival rate in the offspring of rabbits receiving the highest dose (15 mg/kg/day; equal to 1.4 times the human dose).

Hepatic injury: Since transaminase abnormalities, cholestatic jaundice, hepatic necrosis, and hepatic encephalopathy have been reported with the use of flutamide, periodic liver function tests should be considered. (See ADVERSE REACTIONS section.) Appropriate laboratory testing should be done at the first symptom/sign of liver dysfunction (e.g., pruritus, dark urine, persistent anorexia, jaundice, right upper quadrant tenderness or unexplained "flu-like" symptoms). If the patient has jaundice or laboratory evidence of liver injury, in the absence of biopsy-confirmed liver metastases, EULEXIN therapy should be discontinued or the dosage reduced. The hepatic injury is usually reversible after discontinuation of therapy and in some patients, after dosage reduction. However, there have been reports of death following severe hepatic injury associated with use of flutamide.

PRECAUTIONS

Information for Patients: Patients should be informed that EULEXIN Capsules and the drug used for medical castration should be administered concomitantly, and that they should not interrupt their dosing or stop taking these medications without consulting their physician.

Laboratory Tests: See WARNINGS, Hepatic Injury above. Drug Interactions: Interactions between EULEXIN Capsules and leuprolide have not occurred. Increases in prothrombin time have been noted in patients receiving long-term warfarin therapy after flutamide was initiated. Therefore close monitoring of prothrombin time is recommended and adjustment of the anticoagulant dose may be necessary when EULEXIN Capsules are administered concomitantly with warfarin.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No carcinogenicity studies were performed with flutamide. However, daily administration of flutamide to rats for 52 weeks at doses of 30, 90, or 180 mg/kg/day (approximately 3, 8, or 17 times the human dose) produced testicular interstitial cell adenomas at all doses.

Flutamide did not demonstrate DNA modifying activity in the Ames Salmonella/microsome Mutagenesis Assay. Dominant lethal tests in rats were negative.

Reduced sperm counts were observed during a six-week study of flutamide monotherapy in normal human volunteers.

Flutamide did not affect estrous cycles or interfere with the mating behavior of male and female rats when the drug was administered at 25 and 75 mg/kg/day prior to mating. Males treated with 150 mg/kg/day (30 times the minimum effective antiandrogenic dose) failed to mate; mating behavior returned to normal after dosing was stopped. Conception rates were decreased in all dosing groups. Suppression of spermatogenesis was observed in rats dosed for 52 weeks at approximately 3, 8, or 17 times the human dose and in dogs dosed for 78 weeks at 1.4, 2.3, and 3.7 times the human dose.

Pregnancy: Pregnancy Category, D. See WARNINGS section.

ADVERSE REACTIONS

The following adverse experiences were reported during a multicenter clinical trial comparing flutamide + LHRH agonist versus placebo + LHRH agonist.

The most frequently reported (greater than 5%) adverse experiences during treatment with EULEXIN Capsules in combination with a LHRH agonist are listed in the table below. For comparison, adverse experiences seen with a LHRH agonist and placebo are also listed in the following table.

	(n=294) Flutamide + LHRH-agonist	(n=285) Placebo + LHRH-agonist
Hot Flashes	61	57
Loss of Libido	36	31
Impotence	33	29
Diarrhea	12	4
Nausea/Vomiting	11	10
Gynecomastia	9	11
Other	7	9
Other GI	6	4

As shown in the table, for both treatment groups, the most frequently occurring adverse experiences (hot flashes, impotence, loss of libido) were those known to be associated with low serum androgen levels and known to occur with LHRH-agonists alone.

The only notable difference was the higher incidence of diarrhea in the flutamide + LHRH-agonist group (12%), which was severe in five percent as opposed to the placebo + LHRH-agonist (4%), which was severe in less than one percent.

In addition, the following adverse reactions were reported during treatment with flutamide + LHRH-agonist: No causal relatedness of these reactions to drug treatment has been made, and some of the adverse experiences reported are those that commonly occur in elderly patients.

Cardiovascular System: hypertension in 1% of patients. Central Nervous System: CNS (drowsiness/confusion/depression/anxiety/nervousness) reactions occurred in 1% of patients.

Gastrointestinal System: other GI disorders occurred in 6% of patients.

Hematopoietic System: anemia occurred in 6%, leukopenia in 3%, and thrombocytopenia in 1% of patients.

Liver and Biliary System: hepatitis and jaundice in less than 1% of patients.

Skin: irritation at the injection site and rash occurred in 3% of patients.

Other: edema occurred in 4%, genitourinary and neuromuscular symptoms in 2%, and pulmonary symptoms in less than 1% of patients.

In addition, the following spontaneous adverse experiences have been reported during the marketing of flutamide: hemolytic anemia, macrocytic anemia, methemoglobinemia, photosensitivity reactions (including erythema, ulceration, bullous eruptions, and epidermal necrosis) and urine discoloration. The urine was noted to change to an amber or yellow-green appearance which can be attributed to the flutamide and/or its metabolites. Also reported were cholestatic jaundice, hepatic encephalopathy, and hepatic necrosis. The hepatic conditions were usually reversible after discontinuing therapy; however, there have been reports of death following severe hepatic injury associated with use of flutamide.

Abnormal Laboratory Test Values: Laboratory abnormalities including elevated SGOT, SGPT, bilirubin values. SGGT, BUN and serum creatinine have been reported.

OVERDOSAGE

In animal studies with flutamide alone, signs of overdose included hypoactivity, piloerection, slow respiration, ataxia, and/or lacrimation, anorexia, tranquilization, emesis, and methemoglobinemia.

Clinical trials have been conducted with flutamide in doses up to 1500 mg per day for periods up to 36 weeks with no serious adverse effects reported. Those adverse reactions reported included gynecomastia, breast tenderness and some increases in SGOT. The single dose of flutamide ordinarily associated with symptoms of overdose or considered to be life-threatening has not been established.

Since flutamide is highly protein bound, dialysis may not be of any use as treatment for overdose. As in the management of overdose with any drug, it should be borne in mind that multiple agents may have been taken. If vomiting does not occur spontaneously, it should be induced if the patient is alert. General supportive care, including frequent monitoring of the vital signs and close observation of the patient, is indicated.

DOSAGE AND ADMINISTRATION

The recommended dosage is two capsules three times a day at eight hour intervals for a total daily dosage of 750 mg.

HOW SUPPLIED

EULEXIN Capsules, 125 mg, are available as opaque, two-toned brown capsules, imprinted with "Schering 525". They are supplied as follows:

NDC 0085-0525-04—Bottles of 100

NDC 0085-0525-05—Bottles of 500

NDC 0085-0525-03—Unit Dose packages of 100 (10 × 10's)

Store between 2° and 30°C (36° and 86°F).

Protect the unit dose packages from excessive moisture.

Rev. 4/91

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Shown in Product Identification Section, page 427

FULVICIN® P/G

[ful'vī-sin]

brand of ultramicrosize griseofulvin.

Tablets, USP

DESCRIPTION

FULVICIN P/G Tablets contain ultramicrosize crystals of griseofulvin, an antibiotic derived from a species of *Penicillium*. Griseofulvin crystals are partly dissolved in polyethylene glycol 6000 and partly dispersed throughout the tablet matrix.

Each FULVICIN P/G Tablet contains 125 mg or 250 mg griseofulvin, ultramicrosize.

The inactive ingredients for FULVICIN P/G Tablets, 125 or 250 mg, include: corn starch, lactose, magnesium stearate, PEG, and sodium lauryl sulfate.

ACTIONS

Microbiology: Griseofulvin is fungistatic with *in vitro* activity against various species of *Microsporum*, *Epidermophyton*, and *Trichophyton*. It has no effect on bacteria or on other genera of fungi.

Human Pharmacology: Following oral administration, griseofulvin is deposited in the keratin precursor cells and has a greater affinity for diseased tissue. The drug is tightly bound to the new keratin which becomes highly resistant to fungal invasions.

The efficiency of gastrointestinal absorption of ultramicrosize crystalline griseofulvin is approximately one- and one-half times that of the conventional microsize griseofulvin. This factor permits the oral intake of two-thirds as much ultramicrosize crystalline griseofulvin as the microsize form. However, there is currently no evidence that this lower dose confers any significant clinical differences with regard to safety and/or efficacy.

INDICATIONS

FULVICIN P/G Tablets are indicated for the treatment of ringworm infections of the skin, hair, and nails, namely: tinea corporis, tinea pedis, tinea cruris, tinea barbae, tinea capitis, tinea unguium (onychomycosis) when caused by one or more of the following genera of fungi: *Trichophyton*, *Microsporum*, *Trichophyton tonsurans*, *Trichophyton mentagrophytes*, *Trichophyton interdigitale*, *Trichophyton verrucosum*, *Trichophyton megninii*, *Trichophyton gallinae*, *Trichophyton crateriforme*, *Trichophyton sulphureum*, *Trichophyton leucoleinii*, *Microsporum audouinii*, *Microsporum canis*, *Microsporum gypseum*, and *Epidermophyton floccosum*.

Note: Prior to therapy, the type of fungi responsible for the infection should be identified.

The use of this drug is not justified in minor or trivial infections which will respond to topical agents alone.

Griseofulvin is not effective in the following: bacterial infections, candidiasis (moniliasis), histoplasmosis, actinomycosis, sporotrichosis, chromoblastomycosis, coccidioidomycosis, North American blastomycosis, cryptococcosis (torulosis), tinea versicolor, and nocardiosis.

CONTRAINDICATIONS

This drug is contraindicated in patients with porphyria, atocellular failure, and in individuals with a history of hypersensitivity to griseofulvin.

Rare cases of conjoined twins have been reported in patients taking griseofulvin during the first trimester of pregnancy. Griseofulvin should not be prescribed to pregnant patients or to women contemplating pregnancy.

WARNINGS

Prophylactic Usage: Safety and efficacy of griseofulvin prophylaxis of fungal infections have not been established.

Animal Toxicology: Chronic feeding of griseofulvin at levels ranging from 0.5-2.5% of the diet, resulted in the development of liver tumors in several strains of mice, particularly in males. Smaller particle sizes result in an enhanced effect. Lower oral dosage levels have not been tested. Simultaneous administration of relatively small doses of griseofulvin once a week during the first three weeks of life have been reported to induce hepatoma in mice. They have also been reported to induce hepatoma in rats. In rats, hepatomas, mostly adenomas but some carcinomas, have been reported in male rats receiving griseofulvin at levels of 1.0%, and 0.2% of the diet, and in female rats receiving two higher dose levels. Although studies in other

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*Editor, and Chairman
of the Editorial Board*

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Norethynodrel and Mestranol Tablets

Enovid (Searle); Enovid E (Searle)

Uses—Became famous the world over as an oral contraceptive and has been dubbed "The Pill." See page 932. For the effects of the separate components, see *Norethynodrel* (page 935) and *Mestranol* (page 931). The contraceptive action is not entirely the result of prevention of ovulation; the endometrium is rendered inhospitable to the fertilized ovum, so that nidation cannot take place. In addition to oral contraceptive use, preparations containing 5 mg or more of norethynodrel may be used in the treatment of dysmenorrhea and menorrhagia and to produce cyclic withdrawal bleeding.

The side effects are those of the separate components as well as of combination contraceptives (page 937). The most frequent effects are bleeding irregularities, nausea, and vomiting in about 1/4 of all users, breast fullness, chloasma, headache, weakness, dizziness, and diarrhea. The effects diminish with continual use. Sometimes fluid retention and acne occur. Cholestatic jaundice occurs rarely. It is uncertain whether the mixture increases the incidence of thrombophlebitis.

Dose—For contraception, norethynodrel (2.5 or 5 mg) plus mestranol (100 or 75 µg, respectively) daily for 20 or 21 days, starting on the 5th day after menstruation begins. For cyclic therapy in dysmenorrhea, functional uterine bleeding (once it is controlled), premenstrual tension, amenorrhea (once a menses has been accomplished), or idiopathic infertility, one 5- or 10-mg tablet daily for 20 days of each cycle, beginning on the 5th day after menstruation begins; for emergency control of dysfunctional uterine bleeding, one 10-mg tablet 2 or 3 times a day until bleeding is arrested, then once daily

through the 24th day after menstruation began; for endometriosis, one 5- or 10-mg tablet daily for 2 weeks, beginning on the 5th day after menstruation begins, increasing in dosage every 2 weeks until 20 mg a day is being given, to be continued for 6 to 9 months; to delay menstruation, one to two 10-mg tablets daily, beginning at least 1 week in advance of the expected menstruation; to advance menstruation, one 5- or 10-mg tablet for 10 days, beginning on the 5th day after the start of menstruation.

Dosage Forms—Tablets: Norethynodrel/Mestranol, 2.5/100, 5/75, and 9.85/150 mg/µg.

Norgestrel—page 935.

Norgestrel and Ethinyl Estradiol Tablets

Ovral (Wyeth)

Uses—For combination oral contraception (see page 937). For the actions of the separate components see *Norgestrel* (page 935) and *Ethinyl Estradiol* (page 929). The adverse effects are those of the estrogens (page 926), progestins (page 933), and combinations (page 937).

Dose—500 µg of norgestrel plus 50 µg of ethinyl estradiol or 300 µg of norgestrel plus 30 µg of ethinyl estradiol a day for 21 days, starting on the 5th day of the menstrual cycle.

Dosage Forms—Tablets: Norgestrel/Mestranol, 500/50 and 300/30 µg/µg.

Progesterone—page 936.

The Testicular Hormone

The testis has a dual function, to produce the germ cell (the sperm) and to supply the male hormone (testosterone). Two clearly defined groups of cells are found in the testes; the one group in the tubules produces the sperm, while the other, clustered in between the tubules, consists of interstitial cells. The first or spermatogenic tissue is thought by some investigators to produce an internal secretion, but if this is true, the hormone involved has not yet been identified, nor is there agreement about its existence.

The interstitial cells are the seat of production of a steroid hormone, testosterone, which stimulates and maintains the secondary sex organs; these are the penis, prostate gland, seminal vesicles, vas deferens, and scrotum. It also exerts sustaining effects on the spermatogenic cells, and it stimulates the development of bone, muscle, skin, and hair growth, and emotional responses to produce the characteristic adult masculine traits. This group of combined actions of this hormone is termed *androgenic actions*. Testosterone also antagonizes a number of the effects of estrogens, and is sometimes employed clinically for this purpose. This is especially important in the suppression of metastatic carcinoma of the breast. Since it promotes development of the clitoris, which is an anatomic homologue of the penis, androgens may increase the libido of women.

The naturally occurring androgens (androsterone, testosterone) are derivatives of androstane. Testosterone and its esters (testosterone propionate) and derivatives (methyltestosterone) are the most commonly used androgenic steroids. In addition to their androgenic properties, however, these compounds exert widespread anabolic effects; in attempts to dissociate the virilizing and anabolic properties (for use in women) a number of compounds with high anabolic androgenic ratios have been prepared. However, it has not yet been possible to abolish completely the androgenic effects.

Uses—For substitutional therapy in men who have climacteric symptoms, or in men or youths with hypogonadism (eunuchism, Klinefelter's syndrome). They have been employed to facilitate development of adult masculine characteristics when the adolescent process has been delayed. In

cryptorchidism they may be used adjunctively with gonadotropins. They are also very useful in therapy of patients with hypopituitarism and with Addison's disease. They are of value in the treatment of frigidity and occasionally in impotence. Use of androgens for relief of impotence not associated with evidence of testicular underactivity (psychic causes) is known to be futile in most cases. Low doses of androgens have been used in pituitary dwarfism to accelerate growth, but care must be exercised not to arrest growth by epiphyseal closure.

With estrogens, androgen therapy may be efficacious in the treatment of the menopause. The anabolic effects are possibly of some benefit in the postclimacteric person, and they may retard osteoporosis, although many authorities do not believe that any lasting benefit is achieved. In functional dysmenorrhea androgens may give relief through an antiestrogenic action, although they also are often combined with estrogens to treat this disorder. They may be used to treat endometriosis. They may also be used in the treatment of postpartum breast engorgement and for suppression of lactation.

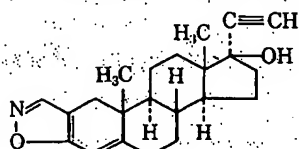
Testosterone and related compounds find widespread application in the palliative treatment of cancer of the breast in women. Its use in men with prostatic cancer, however, is contraindicated.

Side Effects—Androgens cause hirsutism, deepening or hoarseness of the voice, precocious puberty and epiphyseal closure in immature males, increased libido (in both male and female!), priapism, oligospermia, enlargement of the clitoris in the female, flushing, decreased ejaculatory volume, gynecomastia, hypersensitivity, acne, weight gain, edema, and hypercalcemia. Biliary stasis and jaundice occur. There have been a few cases reported of hepatoma following long-term therapy. The 17 α -methylated androgens are more prone to disturb liver function than are the nonsubstituted drugs. Hypercalcemia requires discontinuation of therapy; and edema requires diuretic therapy. Except in the treatment of breast cancer, a reduction in dosage is indicated upon virilization in women.

Administration of androgens to patients on anticoagulant therapy may increase the effect of anticoagulants and thus may require an adjustment of the dose of the latter. Likewise, dosage of insulin or of oral hypoglycemic agents may require adjustment when anabolic androgens are administered to diabetic patients.

Danazol

Pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol, (17 α);
Danocrine (Winthrop)



17 α -Pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol [17230-88-5]
 $C_{22}H_{27}NO_2$ (337.47).

Preparation—Danazol is a derivative of ethisterone (17 α -ethynyltestosterone) in which an isoxazole ring is fused to the 2,3-position of the steroid nucleus. Methods for preparing such steroidal heterocycles have been described by Manson *et al.*, *J Med Chem* 6: 1, 1963, also in US Pat. 3,135,743.

Description—Pale yellow, crystalline powder; melts at about 225°.

Solubility—Practically insoluble in water; sparingly soluble in alcohol.

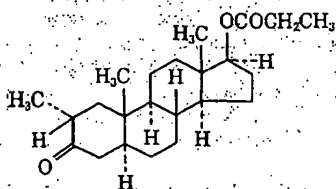
Uses—Danazol is a synthetic androgen with weak androgenic activity and no progestational or estrogenic effects. It is proposed for use only in the treatment of *endometriosis* in patients who do not respond to or cannot tolerate other drug therapy. It has been reported to prevent attacks of hereditary angioedema. Androgenic side effects include acne, edema, mild hirsutism, decrease in breast size, oiliness of the skin and hair, weight gain, and clitoral hypertrophy. Hypoestrogenic manifestations include vasomotor instability, vaginitis with itching, burning and vaginal bleeding, and emotional lability. Structurally similar C-17-alkylated androgens have been reported to cause hepatic injury, including carcinoma. Danazol has been reported to lower serum levothyroxine levels.

Dose—*Oral*, 800 mg, divided into two doses, per day.

Dosage Form—Capsules: 200 mg.

Dromostanolone Propionate

Androstan-3-one, 2-methyl-17-(1-oxopropoxy)-, (2 α ,5 α ,17 β);
Drolban (Lilly)



17 β -Hydroxy-2 α -methyl-5 α -androstan-3-one propionate [521-12-0] $C_{23}H_{36}O_3$ (360.54).

Preparation—Testosterone is reacted with ethyl formate and alkali metal hydride to form 2-(hydroxymethylene)testosterone (I) (*J Am Chem Soc* 76: 552, 1954). Refluxing a benzene suspension of I, methyl iodide, and sodium hydride under nitrogen produces 2-formyl-2-methyltestosterone which is then decarbonylated by passage through a column of alkalized alumina to yield 2 α -methyltestosterone (II). Esterification of II with propionic anhydride in pyridine solution forms II propionate which yields dromostanolone propionate on hydrogenation in the presence of palladium on barium sulfate or various other catalysts. US Pat. 3,118,915.

Description—White to creamy white, crystalline powder; odorless or has a faint odor; melts, with a range of 4°, between 127° and 133°.

Solubility—1 g in 30 ml alcohol, 2 ml chloroform, 20 ml ether; practically insoluble in water.

Uses—An *androgen* similar in its actions to *Testosterone Propionate* (page 943), but it appears to be somewhat less virilizing. Its

use has been restricted to the treatment of metastatic carcinoma of the breast. With respect to efficacy in the regression of the carcinoma, it is about equivalent to testosterone propionate, but its lesser virilizing activity makes dromostanolone advantageous. The drug may improve anemia and the patient's sense of well-being, but these effects are unrelated to regression of the disease.

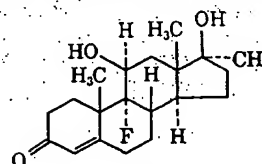
The untoward effects of dromostanolone are mainly those which result from virilization and include facial hair growth, deepening of the voice, acne, and enlargement of the clitoris, which occasionally may give rise to an increase in libido. The virilizing effects develop slowly and may not reach their peak for several months. Fluid retention with edema may occur. Effects on serum calcium are not untoward but rather reflect the success of the drug on osteolytic metastases; if regression occurs, serum calcium falls. Jaundice has not been reported, but the potential exists. Dromostanolone should be used with caution in the presence of liver disease, heart failure, kidney disease, and pregnancy.

Dose—*Intramuscular*, 100 mg 3 times a week for 8 to 12 weeks or for the duration of a remission caused by the drug.

Dosage Forms—Injection: 50 mg/ml, 500 mg/10 ml.

Fluoxymesterone

Androst-4-en-3-one, 9-fluoro-11,17-dihydroxy-17-methyl-, (11 β ,17 β);
Halotestin (Upjohn); Ora-Testril (Squibb)



9-Fluoro-11 β ,17 β -dihydroxy-17-methylandrosta-4-en-3-one [76-43-7] $C_{20}H_{29}FO_3$ (336.45).

Preparation—From 17-methyltestosterone first by introduction of a hydroxyl group at position 11 through oxidation with a micro-organism (such as *Pestalotia* or *Aspergillus*), followed by dehydration, epoxidation and treatment with HF, as for *Betamethasone* (page 902).

Description—White or practically white, odorless, crystalline powder; melts at about 240°, with some decomposition.

Solubility—Practically insoluble in water; sparingly soluble in alcohol; slightly soluble in chloroform.

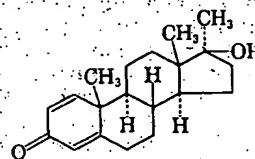
Uses—The same actions, uses, and limitations as androgens (page 939). It is approximately five times more potent than testosterone, and is orally effective. In addition to the side effects of testosterone, fluoxymesterone may cause occasional cholestatic jaundice, gynecostasia, oligospermia after prolonged use, and hypersensitivity. Fluoxymesterone is sometimes combined with an estrogen for treatment of postmenopausal osteoporosis.

Dose—*Oral, replacement therapy*, 1 to 5 mg twice a day; for *anabolic effect* and treatment of *osteoporosis*, 2 to 5 mg twice a day; for metastatic breast cancer in women, 5 to 10 mg 3 times a day; in *hypoplastic or aplastic anemia*, 10 to 40 mg a day; for *postpartum breast engorgement*, 2.5 to 5 mg twice a day for 4 or 5 days postpartum.

Dosage Forms—Tablets: 2.5 and 10 mg.

Methandrostenolone

Androsta-1,4-diene-3-one, 17-hydroxy-17-methyl-, (17 β);
Methandienone; Dianabol (Ciba)



17 β -Hydroxy-17-methylandrosta-1,4-dien-3-one [72-63-9] $C_{20}H_{28}O_2$ (300.44).

Preparation—*Methyltestosterone* is dehydrogenated, either by microbial methods or by reaction with selenium dioxide, to create the Δ^1 double bond. US Pat. 2,900,398.

Description—White to off-white crystals or crystalline powder; odorless; melts at about 165°.

Solubility—Insoluble in water; soluble in alcohol, chloroform, glacial acetic acid; slightly soluble in ether.

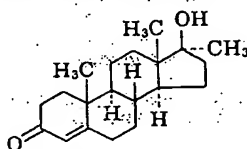
Uses—An androgenic steroid with relatively strong anabolic and weak androgenic activity. Consequently, it is employed mainly to promote nitrogen anabolism and weight gain in cachexia and debilitating diseases and after serious infections, burns, trauma, or surgery. It may relieve pain in certain types of osteoporosis, and it favors the retention of calcium, which assists in arresting the disease. It also helps to relieve pain and to promote a sense of well-being in the arthritides. Side effects include virilization and acne, especially in women and children, sodium retention and edema, and cholestatic jaundice. Methandrostenolone potentiates prothrombopenic anticoagulants and hence may favor hemorrhagic diatheses in persons taking such drugs.

Dose—Oral, initially, 5 mg daily and, for maintenance, 2.5 to 5 mg daily, except up to 50 µg/kg a day in older children and adults with pituitary dwarfism. Continuous therapy should consist in repeated courses of no longer than 6 weeks, separated by intervals of 2 to 4 weeks.

Dosage Forms—Scored Tablets: 2.5 and 5 mg.

Methyltestosterone

Androst-4-en-3-one, 17-hydroxy-17-methyl-, (17β)-;
Metandren (Ciba); Oreton Methyl (Schering)



17β-Hydroxy-17-methylandrost-4-en-3-one [58-18-4] C₂₀H₃₀O₂ (302.46).

Preparation—Readily from dehydroepiandrosterone (prepared from cholesterol) by subjecting it to a Grignard reaction with CH₃MgI followed by an Oppenauer oxidation. The first reaction creates the tertiary carbinol structure at C₁₇, while the second oxidizes the secondary carbinol group at position 3 to carbonyl and causes a rearrangement of the double bond from the 5,6- to the 4,5- position.

Description—White or creamy white crystals or a crystalline powder; odorless, stable in air, but slightly hygroscopic; affected by light; melts between 162° and 167°.

Solubility—Practically insoluble in water; soluble in alcohol, methanol, ether, and other organic solvents; sparingly soluble in vegetable oils.

Uses—The actions, uses, and limitations are the same as those of androgens in general (page 939). Methyltestosterone is effective orally. It is also combined with various estrogens for treatment of menorrhagia, menopausal symptoms, dysmenorrhea, osteoporosis, malnutrition, and to suppress postpartum lactation. In addition to the side effects caused by testosterone, methyltestosterone may cause oligospermia, hypersensitivity with dermatologic manifestations, and a rare type of cholestatic jaundice. It is often stated that virilization in women does not occur unless the dose exceeds 300 mg/month, but virilization can occur with doses considerably less than this.

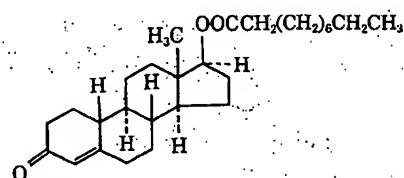
By the buccal route, potency is twice that by the oral route.

Dose—Oral, for replacement therapy, 5 to 20 mg twice a day; for anabolic effects, 10 to 20 mg a day; to prevent postpartum breast engorgement and pain, 40 mg twice a day for 3 to 5 days; for inoperable breast cancer in women, 100 mg twice a day for the duration of improvement or for no longer than 3 months if there is no remission. Buccal, one-half the oral dose.

Dosage Forms—Capsules: 10 mg; Capsules (timed-release): 5 and 10 mg; Tablets: 10 and 25 mg; Tablets (buccal): 5 and 10 mg.

Nandrolone Decanoate

Estr-4-en-3-one, 17-[(1-oxodecyl)oxy]-, (17β)-;
Deca-Durabolin (Organon)



17β-Hydroxyestr-4-en-3-one decanoate [360-70-3] C₂₈H₄₄O₃ (428.65).

Preparation—A dry benzene solution of 17β-hydroxy-estr-4-en-3-one (19-nortestosterone) and pyridine is mixed with a dry benzene solution of decanoyl chloride and the esterification is allowed to proceed overnight in an atmosphere of nitrogen. After washing successively with acid, alkali, and water, the solvent is evaporated and the crude ester is recrystallized from petroleum ether or some other suitable solvent. US Pat. 2,998,423.

Description—Fine, white to creamy white, crystalline powder; odorless or may have a slight odor; melts between 33° and 37°.

Solubility—Soluble in chloroform, alcohol, acetone, and vegetable oils; practically insoluble in water.

Uses—The actions and uses are the same as those of Nandrolone Phenpropionate. Oil solutions of the decanoate have a duration of action 3 to 4 times longer than that of the phenpropionate.

Dose—Intramuscular, adult, anabolic, 50 to 100 mg every 3 to 4 weeks; children 2 to 13 years of age, 12.5 to 25 mg every 2 to 4 weeks. In metastatic breast carcinoma and refractory anemias the dose may be 100 to 200 mg a week, depending on the response.

Dosage Forms—Injection: 50 mg/ml, 100 mg/ml.

Nandrolone Phenpropionate

Estr-4-en-3-one, 17-(1-oxo-3-phenylpropoxy)-, (17β)-;
Durabolin (Organon)

17β-Hydroxyestr-4-en-3-one hydrocinnamate [62-90-8] C₂₇H₃₄O₃ (406.57).

For the structure of the steroid moiety, see Nandrolone Decanoate.

Preparation—19-Nortestosterone is esterified with hydrocinnamoyl chloride by the method described for Nandrolone Decanoate.

Description—Fine, white to creamy white, crystalline powder having a slight characteristic odor; melts between 95° and 99°.

Solubility—Practically insoluble in water; soluble in alcohol (1 g in 2 ml), chloroform, dioxane, and vegetable oils.

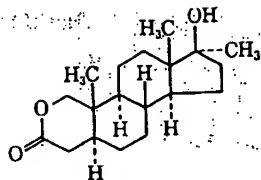
Uses—A synthetic androgen with actions intermediate to those of Testosterone and Norethandrolone. Although nandrolone phenpropionate is less androgenic than testosterone in doses which exert anabolic actions, virilization may occur after high doses or during chronic administration. Indeed, the androgenic virilizing actions are sought in the treatment with this agent of inoperable breast cancer in women. Nandrolone phenpropionate is mainly used in the treatment of chronic wasting diseases and conditions in which negative nitrogen balance exists. Low doses may accelerate growth of children with retarded growth without excessively accelerating bone age; higher doses accelerate bone maturation more than body growth. The phenylpropionate ester moiety confers a long duration of action to suspensions in oil injected intramuscularly. The potential side effects are those of testosterone. Nandrolone phenpropionate does not appear to cause cholestatic jaundice, probably because it lacks an alkyl group on carbon 17.

Dose—Intramuscular, adults, anabolic, 25 to 50 mg each week; inoperable breast cancer and refractory anemias, 50 to 100 mg a week; postpartum breast engorgement, 25 to 50 mg a day for 3 or 4 days. For children 2 to 13 years of age, 12.5 to 25 mg every 2 to 4 weeks; for infants, 1 mg/kg of body weight every 2 to 4 weeks.

Dosage Forms—Injection: 25 mg/ml, 100 mg/ml.

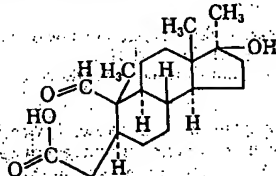
Oxandrolone

2-Oxaandrost-3-one, 17-hydroxy-17-methyl-, (5α,17β)-;
Anavar (Searle)

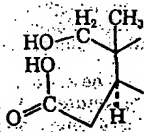


17β-Hydroxy-17-methyl-2-oxa-5α-androstan-3-one [53-39-4]
C₁₉H₃₀O₃ (306.44)

Preparation—Methylidihydrotestosterone is converted into the corresponding 1,2-dehydro compound by bromination followed by dehydrobromination. Ring A is then ruptured through ozonization and subsequent hydrolysis to yield the aldehyde-acid (I). Reduction of the formyl group in I yields the expected hydroxy acid implied in the partial structure (II) which is lactonized to oxandrolone.



(I)



(II)

Description—White, odorless, crystalline powder; stable in air but darkens when exposed to light; melts at about 225°.

Solubility—1 g in 5200 ml water, 57 ml alcohol, <5 ml chloroform, 860 ml ether, 69 ml acetone.

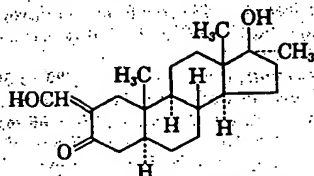
Uses—Although not strictly speaking a steroid, its configuration is that of a 17-methyl androgenic steroid. Its anabolic actions are strong relative to its androgenic actions. Consequently, it is used in the treatment of chronic wasting diseases and conditions in which negative nitrogen balance exists. In children with retarded growth, low doses of oxandrolone may increase height and weight without increasing bone age to a comparable degree, but large doses may advance bone age and cause closure of the epiphyses. The drug favors calcium retention and bone reconstruction and hence may be used to treat osteoporosis; however, in females it is probably usually better to use estrogens for that purpose. The drug may cause virilization, especially in children or in adults if the recommended doses are exceeded. The potential toxicity is that of the androgens but the incidence and severity are less than with testosterone. Oxandrolone may adversely affect liver function tests, and the possibility of cholestatic jaundice must be kept in mind. Leukopenia has also been reported. It is contraindicated in prostatic cancer, breast cancer in some women, pregnancy, nephrosis, and premature and newborn infants.

Dose—Adults, initially 2.5 to 5 mg 2 to 4 times a day, then 2.5 to 5 mg a day for maintenance, not to be taken for more than 3 months in any one course; for children, 250 μg/kg a day, repeated as indicated.

Dosage Form—Tablets: 2.5 mg.

Oxymetholone

Androstan-3-one, 17-hydroxy-2-(hydroxymethylene)-17-methyl-, (5α,17β)-; Adroyd (Parke-Davis); Anadrol (Syntex).



17β-Hydroxy-2-(hydroxymethylene)-17-methyl-5α-androstan-3-one [434-07-1] C₂₁H₃₂O₃ (332.48)

Preparation—17β-Hydroxy-17-methylandrostan-3-one (17-methylidihydrotestosterone) is reacted with ethyl formate and sodium hydroxide by stirring the mixture under nitrogen for several hours thus forming the 2-(sodoxymethylene) derivative. Treatment of the washed sodium compound with cold dilute hydrochloric acid liberates

the oxymetholone which may be purified by recrystallization from ethyl acetate. *J Am Chem Soc* 81: 427, 1959.

Description—White to creamy white crystals or crystalline powder; odorless and stable in air; tautomeric in nature and can exist as either tautomer or as a mixture of both, the exact composition depending on solvent and rate of crystallization; melts between 172° and 180°.

Solubility—1 g in >10,000 ml water, 40 ml alcohol, 5 ml chloroform, 82 ml ether, 14 ml dioxane.

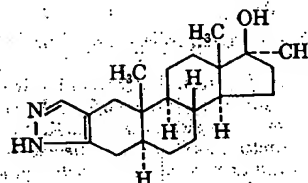
Uses—An androgenic steroid with relatively greater anabolic activity than androgenic activity. Consequently, it is mainly employed to promote nitrogen anabolism and weight gain in cachexia and debilitating diseases and after serious infections, burns, trauma or surgery. It may relieve pain in certain types of osteoporosis, and it promotes calcium retention, so that the condition of the bone may improve. It may be used for its erythropoietic effects in the treatment of hypoplastic and aplastic anemias. Side effects include nausea, vomiting, anorexia, burning of the tongue, increased or decreased libido, acne, suppression of gonadotropin secretion, virilization (especially in women and children), gynecomastia in males, oligospermia, sodium retention and edema, abnormal liver function tests, cholestatic jaundice, decrease in several clotting factors, and hemorrhagic diathesis in the presence of anticoagulants.

Dose—Anabolic, adults, 5 to 10 mg (occasionally 30 mg) a day, preferably for 3 weeks and never longer than 13 weeks/course, with rests of 2 to 4 weeks between courses; in prepubertal children, 2.5 to 5 mg a day, for not more than 30 days/course. In hypoplastic or aplastic anemia or myelofibrosis, children and adults, usually 1 to 2 mg/kg of body weight a day, but higher doses, up to 5 mg/kg, may be required.

Dosage Forms—Tablets: 5, 10, and 50 mg.

Stanozolol

2H-Androst-2-eno[3,2-c]pyrazol-17-ol, 17-methyl-, (5α,17β)-; Winstrol (Winthrop).



17-Methyl-2H-5α-androst-2-eno[3,2-c]pyrazol-17β-ol [10418-03-8]
C₂₁H₃₂N₂O (328.50)

Preparation—17-Methyl-5α-androstan-17β-ol-3-one is converted into its 2-formyl derivative which is then condensed with hydrazine hydrate. US Pat. 3,030,358.

Description—Nearly colorless, odorless, crystalline powder; exists in two forms: needles, melting at about 155°, and prisms, melting at about 235°.

Solubility—1 g in >1000 ml water, 41 ml alcohol, 74 ml chloroform, 370 ml ether.

Uses—An androgenic steroid with relatively strong anabolic and weak androgenic activity. Consequently, it is employed mainly to promote nitrogen anabolism and weight gain in cachexia and debilitating diseases and after serious infections, burns, trauma, or surgery. Although it may relieve pain in certain types of osteoporosis, it apparently does not affect bone density. It may have an erythropoietic effect in hypoplastic and aplastic anemias.

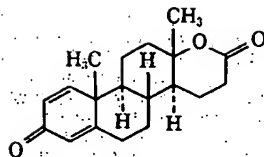
Side effects include increased or decreased libido, virilization (especially in women and children), sodium retention and edema; hypercalcemia; insomnia, restlessness, chills, hemorrhage in patients on anticoagulants, acne, and hepatic dysfunction. Potentially any of the side effects of Testosterone may occur.

Dose—Adults and children over 6 years of age, 2 mg 3 times a day; children under 6 years of age, 1 mg twice a day.

Dosage Form—Scored Tablets: 2 mg.

Testolactone

D-Homo-17a-oxaandrosta-1,4-diene-3,17-dione; Teslac (Squibb)



13-Hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid δ -lactone [968-93-4] $C_{19}H_{24}O_3$ (300.40).

Preparation—By microbial transformation of progesterone, testosterone, and various other steroidal substances. US Pat. 2,744,120; *J Org Chem* 30: 760, 1965.

Description—White to off-white, practically odorless, crystalline powder; stable in light, air, and normal temperatures; melts at about 218° .

Solubility—Slightly soluble in water and benzyl alcohol; soluble in alcohol and chloroform; insoluble in ether and solvent hexane.

Uses—Although structurally related to the androgens, it is essentially devoid of androgenic activity in therapeutic doses. It is used in the adjunctive and palliative treatment of inoperable breast cancer in women. It is not known whether the antineoplastic effect is the result of a unique cytotoxic action or whether its mechanism is that of the androgens in general. Remissions occur in approximately 15% of cases.

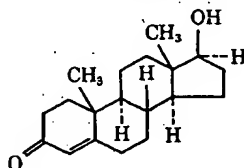
Except possibly for hypercalcemia, the side effects are not those of the androgens. There may be mild pain, irritation, and inflammation at the site of injection. Other reported side effects are nausea, vomiting, aches, myalgia, arthralgia, edema of the extremities, paresthesias, maculopapular rash, hypertension; these all subside without discontinuation of the drug; and it is not always clear to what extent the drug or the disease is responsible.

Dose—*Intramuscular*, 100 mg 3 times a week; *oral*, usually 250 mg 4 times a day.

Dosage Forms—Sterile Suspension: 100 mg/ml; Tablets: 50 and 250 mg.

Testosterone

Androst-4-en-3-one, 17-hydroxy, (17 β)-;
Oreton (Schering)



17 β -Hydroxyandrost-4-en-3-one [58-22-0] $C_{19}H_{28}O_2$ (288.43).

Preparation—First isolated in crystalline form by Laquer in 1935 who obtained it from animal testes. Although small amounts of testosterone may be extracted from testicular material, the synthetic commercial supply is derived from cholesterol. The key intermediate in the synthesis is dehydroepiandrosterone which can be treated further, by either chemical or microbiological processes, to yield testosterone. US Pat. 2,236,574.

Description—White or slightly creamy white crystals or crystalline powder; odorless; stable in air; melting range 153° to 157° .

Solubility—Practically insoluble in water; 1 g in about 6 ml of dehydrated alcohol, 1 ml chloroform, 100 ml ether; soluble in vegetable oils.

Uses—See the general statement, page 939. Testosterone is not effective orally because it is destroyed in the liver on absorption. Its plasma half-life is slightly less than 2 hours.

Dose—*Intramuscular*, in various male hypogonadal states, 10 to 50 mg 2 or 3 times a week; for dysmenorrhea and premenstrual tension, 25 mg 2 times a week for 2 weeks or 25 mg a few days before menstruation; for dysfunctional uterine bleeding, 25 to 50 mg once or twice a week for 2 weeks, then $\frac{1}{2}$ this amount as required; in endometriosis, 25 mg 6 times a week for 4 weeks, beginning midcycle; to prevent postpartum breast engorgement, 25 to 50 mg once a day for 3 or 4 days; in the menopause, 10 to 25 mg 1 or 2 times a week; for frigidity, 10 to 25 mg 2 or 3 times a week for 6 to 8 weeks; in metastatic breast cancer, 25 to 100 mg 3 times a week for low-grade and up to 1 g a week in high-grade malignancy; for anabolic effect, 10 to 25 mg

3 to 7 times a week. *Subcutaneous implantation*, for male hypogonadism, 150 to 450 mg (2 to 6 pellets) every 4 to 6 months.

Dosage Forms—Pellets: 75 mg; Sterile Suspension: 25, 50, and 100 mg/ml.

Testosterone Cypionate

Androst-4-en-3-one, 17-(3-cyclopentyl-1-oxopropoxy)-, (17 β)-;

Testosterone Cyclopentylpropionate USP XVI;

Depo-Testosterone Cypionate (Upjohn)

Testosterone cyclopentanepropionate [58-20-8] $C_{27}H_{40}O_3$ (412.61).

For the structure of the base, see *Testosterone*.

Preparation—Testosterone is esterified by interaction with 3-cyclopentylpropionyl chloride [$C_5H_{11}CH_2CH_2COCl$] in the presence of pyridine. The solid ester is recovered by pouring the reaction mixture into cold 6N sulfuric acid, extracting the crude ester with isopropyl ether, washing the extract with water, drying the solution, removing the solvent, and crystallizing from hexane.

Description—White or creamy white, crystalline powder which is odorless or has a slight odor and is stable in air; melts between 98° and 104° .

Solubility—Insoluble in water; freely soluble in alcohol, chloroform, dioxane, and ether; soluble in vegetable oils.

Uses—The actions, uses, and limitations are the same as for androgens in general (see the general statement, page 939) but the cypionate has a much longer duration of action than testosterone when administered intramuscularly in oil. It is not used in the female except to treat postmenopausal osteoporosis and breast cancer. Virilism in women is likely if the dose exceeds 150 mg a month.

Dose—*Intramuscular*, in oil, in various male hypogonadal states and impotence, 200 to 400 mg every 3 to 6 weeks initially and 4 to 6 weeks for maintenance once a response occurs; for oligospermia, 100 to 200 mg every 3 to 6 weeks or 200 mg each week for 6 to 10 weeks, after which a rebound surge in sperm production may occur; for anabolic effects and osteoporosis, 200 to 400 mg every 3 to 4 weeks; for metastatic breast cancer, 200 to 400 mg every 2 weeks or longer.

Dosage Forms—Injection: 50, 100, and 200 mg/ml, 500 mg and 1 and 2 g/10 ml.

Testosterone Enanthate

Androst-4-en-3-one, 17-[(1-oxoheptyl)oxy]-, (17 β)-;
Delatestryl (Squibb)

Testosterone heptanoate [315-37-7] $C_{26}H_{40}O_3$ (400.60).

For the structure of the base, see *Testosterone*.

Preparation—A solution of enanthic acid in benzene is refluxed for about one hour after which it is allowed to cool, testosterone is added, and the mixture is refluxed for about 21 hours. The resulting light brown solution is cooled, extracted with a sodium hydroxide solution to remove surplus enanthic acid, washed with water, and dried over magnesium sulfate. After removal of solvent, the crude ester is purified by molecular distillation.

Description—White or creamy white, crystalline powder; odorless or has a faint odor characteristic of enanthic acid; melts between 34° and 39° , the initial temperature of the bath not exceeding 20° .

Solubility—Insoluble in water; 1 g in about 0.3 ml ether; soluble in vegetable oils.

Uses—The actions, uses, and limitations are the same as those of *Testosterone Cypionate*. The effects of a single intramuscular injection may last 3 to 4 weeks.

Dose—*Intramuscular*, for replacement in male hypogonadal states and for impotence, 100 to 400 mg every 4 to 6 weeks; for oligospermia, 100 to 200 mg every 4 to 6 weeks or 200 mg each week for 6 to 12 weeks, after which a rebound surge in sperm production may occur; anabolic (antianemic), 200 to 600 mg (or 3 to 10 mg/kg of body weight) once a week for several months; osteoporosis, 200 to 400 mg once every 4 weeks.

Dosage Forms—Injection: 200 mg/ml, 500 mg and 1 g/5 ml, 1 and 2 g/10 ml.

Testosterone Propionate

Androst-4-en-3-one, 17-(1-oxopropoxy)-, (17 β)-;
Oreton Propionate (Schering)

17 β -Hydroxyandrost-4-en-3-one propionate [57-85-2] $C_{22}H_{32}O_3$ (344.49).

For the structure of the base, see *Testosterone*.

Preparation—Readily from testosterone by refluxing with propionic anhydride.

Description—White or creamy white crystals or crystalline powder; odorless and stable in air; melts between 118° and 123°.

Solubility—Insoluble in water; freely soluble in alcohol, dioxane, ether, and other organic solvents; soluble in vegetable oils.

Uses—The actions, uses, and limitations are the same as those of androgens. Intramuscular injection of the propionate provides a somewhat more intense action than with testosterone, but the duration of action is somewhat shorter, even though the half-life is about 4 hours. The parenteral route is not suited to long-term treatment. The other esters of testosterone and synthetic congeners have considerably diminished the importance of the propionate.

Dose—*Intramuscular*, for various male hypogonadal states, 10 to 25 mg 2 to 5 times a week; for postpubertal cryptorchidism, 15 mg twice a day; for anabolic effects, 5 to 10 mg a day; to prevent postpartum breast engorgement, 25 to 50 mg a day for 3 or 4 days, starting at delivery; for metastatic breast cancer in women, 100 mg 3 times a week. *Buccal*, in male hypogonadism, 2.5 to 10 mg twice a day in divided doses; to prevent postpartum breast engorgement, 20 mg twice a day for 3 to 5 days, starting shortly after delivery; in metastatic breast cancer, 50 mg twice a day for as long as improvement is maintained or for 3 months if no improvement obtains.

Dosage Forms—Injection: 10, 25, 50, and 100 mg/ml; Tablets, Buccal: 10 mg.

Other Androgenic Hormones

Calusterone [17 β -Hydroxy-7 β ,17-dimethylandrost-4-en-3-one [17021-26-0], $C_{21}H_{32}O_2$ (316.48); Methosarb (*Upjohn*)]—Reported to be

synthesized by reduction of 6-dehydro-7,17 α -dimethyltestosterone. White to off-white, odorless, tasteless, crystalline powder; stable in light and air; melts between 128° and 134°. Insoluble in water; freely soluble in alcohol. **Uses**: Has very weak androgenic actions yet is relatively potent in the palliation of metastatic breast carcinoma. Whether it has a unique cytotoxic action or whether the antineoplastic effect has the same mechanism as other androgens is unknown. It is not used for androgenic therapy. Signs of virilization, such as deepening of the voice, acne, and facial hair growth occur in 20 to 25% of users. Edema, clitoral enlargement, and other androgenic effects occur occasionally but are mild. Hypercalcemia has been reported. Although some liver function tests may be altered, cholestatic jaundice is rare. Nausea and vomiting occur in 5 to 10% of patients. **Dose**: 150 to 300 mg a day. **Dosage Form**: Tablets: 50 mg.

Ethylestrenol [17 α -Ethylestr-4-en-17-ol $C_{20}H_{32}O$ (288.46); *Manibolin* (*Organon*)]—A solution of 3-ethoxy-17 α -ethylestradiol in ether is reacted in the cold with dry ethylamine to which lithium has been added. Following solvent extraction, the crude dry product is distributed between petroleum ether and 70% methanol. The petroleum ether layer is separated and evaporated to yield the ethylestrenol. US Pat. 3,112,328. White to creamy white, crystalline powder that is odorless and tasteless; unstable in heat and light; melts between 83° and 95°. Freely soluble in alcohol; soluble in chloroform; practically insoluble in water. **Uses**: An anabolic steroid related to the androgens. It promotes tissue building, a renewal of vigor, a feeling of well-being, and bone matrix reconstruction. Consequently, there is an increase in appetite and body weight. It is used in treating the wasting diseases to facilitate convalescence from prolonged illness, and to arrest osteoporosis. It is also used to antagonize certain catabolic effects of corticosteroid therapy. Ethylestrenol potentially has all the side effects of *Testosterone* (page 943), except that they are generally of lower incidence and weaker. Its most serious side effect is cholestatic jaundice, in common with other anabolic steroids. Although its androgenic actions are weak, the drug may induce withdrawal bleeding and amenorrhea in women, and it is contraindicated in prostatic carcinoma. **Dose**: Oral, 4 mg once a day for up to 6 weeks; after a 4-week pause, an additional course may be administered if indicated. For children, 1 to 3 mg a day.

**Suppression of Spermatogenesis by Testosterone in Adult Male Rats:
Effect on Fertility, Pregnancy Outcome and Progeny¹**

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ABSTRACT

The relationship between decreasing spermatogenic activity and fertility, pregnancy outcome and the progeny is poorly understood. To study this relationship a model where testosterone is given by a sustained release device is used. Adult male Sprague-Dawley rats received empty or testosterone-filled implants measuring 0.5, 1.0, 2.0, 3.0, 4.0 and 8.0 cm. On Day 90 and again on Day 104 each male was exposed to two females in proestrus. Twenty days later the females were killed. Corpora lutea, implantation sites, resorptions and live normal and abnormal fetuses were counted. Sperm counts in the caput-corpus region of the epididymis in the 3.0-, 4.0- and 8.0-cm testosterone treatment groups were 12.6%, 3.0% and 29.9% of control, while those in the caudal region were 19.8%, 4.0% and 50.8% of control, respectively. The number of females with spermatozoa in the vagina after breeding was significantly diminished only in animals treated with the 4.0-cm testosterone implants (control, 95.8%; 4.0-cm, 50%) while the number of pregnant females per sperm-positive females was markedly reduced in the females mated with both the 3.0-cm and 4.0-cm testosterone implants (control, 82.6%; 3.0-cm, 10.0%; 4.0-cm, 7.7%). There was no effect on the numbers of corpora lutea, on the incidence of pre- or post-implantation loss, malformations, or on the numbers of pups/litter. Individual animals with a decrease in caudal epididymal spermatozoal reserves to less than 5 million, however, are infertile. A decrease in epididymal spermatozoal reserves mediated by testosterone does not cause an increase in teratogenicity in the resultant progeny.

INTRODUCTION

In the past decade there have been increasing efforts to develop a male contraceptive. Associated with these efforts some major questions have become apparent. Namely, is azoospermia a necessary condition for an effective male contraceptive and are the progeny that may result from oligospermic males normal? These concerns have been clearly underscored in the Guidelines for the Clinical Testing of Contraceptive Drugs (Schaffenburg et al., 1981).

Since the early 1970's it has been established that testosterone administered via polydimethylsiloxane (PDS) capsules can, at appropriate

doses, suppress spermatogenesis (Ewing et al., 1973; Reddy and Prasad, 1973). The ability of this endogenous compound to induce azoospermia as opposed to oligospermia is species-specific (Ewing and Robaire, 1978). In rats, monkeys and man, testosterone administration results in oligospermia (Ewing et al., 1976; Steinberger et al., 1978; Swerdloff et al., 1978; Robaire et al., 1979; Lobl et al., 1983). Thus, due to the inability of testosterone capsules to consistently bring about azoospermia, a condition thought to be essential for a contraceptive formulation, numerous efforts have been made combining testosterone with other compounds such as estradiol (Robaire et al., 1979; Kuhl et al., 1981), progestagens (Brenner et al., 1977; Schearer, 1978), and luteinizing hormone-releasing hormone (LHRH) analogs (Heber and Swerdloff, 1980; Doelle et al., 1983). Though it is possible to attain azoospermia in some species with some of these combinations, the two basic questions of the necessity of azoospermia and of the possible adverse effects of

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hormone-induced oligospermia on any potential progeny are unresolved.

The present study was designed to evaluate the effect of graded decreases in spermatogenesis as induced by testosterone on fertility, pregnancy outcome and the progeny. We have found that: 1) though no treatment decreased the mean caudal epididymal spermatozoal reserved to less than 5 million, individual animals having a decrease in caudal epididymal spermatozoal reserved below this number are infertile, and 2) a decrease in epididymal spermatozoal reserves does not cause an increase in teratogenicity in the resultant progeny.

MATERIALS AND METHODS

Treatment of Males

Adult male Sprague-Dawley rats (300–350 g) were obtained from Charles River Ltd. Canada (St. Constant, Quebec), maintained on a 14L:10D cycle and provided food and water ad libitum. After 1 wk habituation period the animals were randomly assigned to one of seven treatment groups ($n=6$). Each animal was implanted with a subdermal polydimethylsiloxane (PDS) capsule(s). The capsules were made from PDS tubing having an o.d. of 3.18 mm and an i.d. of 1.98 mm (Dow Corning catalogue #602–305, Midland, MI), medical adhesive silicone type A (Dow-Corning catalogue #891) and filled with testosterone (Steraloids, Wilton, NH). The procedure for preparing the capsules has previously been described (Stratton et al., 1973). Under ether anesthesia, control animals were implanted with empty capsules measuring 4.0 cm and the six testosterone treatment groups received implants of the steroid-filled capsules measuring 0.5, 1.0, 2.0, 3.0, 4.0 and 8.0 (2 X 4.0) cm, respectively. The testosterone release rate was $\sim 30 \mu\text{g} \cdot \text{cm} \cdot \text{day}$ (Robaire and Zirkin, 1981). The animals were weighed at weekly intervals.

Fertility Studies

Adult female Sprague-Dawley rats (200–225 g) were obtained from Charles River Ltd. Canada, and were smeared daily to monitor their estrous cycles. Two females in proestrus were mated to each of the males that had been implanted with PDS capsules 90 days before. Each male was mated to a second group of two females in proestrus 14 days after the first mating. Males were killed within a week of the second mating by decapitation. Trunk blood was collected, serum was prepared and stored frozen at -80°C until assayed for testosterone by radioimmunoassay as previously described (Scheer and Robaire, 1980). Testes, epididymides and seminal vesicles were removed, blotted and weighed. The epididymides were divided into caput-corporis and cauda regions; each of the four sections was homogenized (in 0.9% NaCl, 0.1% sodium merthiolate, and 0.5% Triton X-100) and spermatozoal heads were counted to assess tissue content of spermatozoa (Robb et al., 1978).

Analysis of Pregnancy Outcome

The morning after each mating, the number of seminal plugs for each male (the number on the tray beneath the suspended metal grid of the cage or in the female) was counted. Females were examined for the presence of spermatozoa in the vagina. Twenty days after each mating, the females (total number 168) were guillotined. The ovaries were removed and the number of corpora lutea was counted. The uteri were caesarian sectioned and the number of implantations, resorptions and live fetuses were counted. Fetuses were blotted dry, weighed and examined for external malformations and sex. Half of the live fetuses were fixed in absolute ethanol for skeletal staining and examination (Inouye, 1976); and the other half of the fetuses were fixed in Bouin's solution for examination of internal malformations by Wilson's razor blade sections (Wilson, 1965).

Statistical Evaluation

All data were analyzed by one-way analysis of variance (ANOVA) with the new Duncan's multiple range test (Dunnett, 1970) and by chi-square analysis with Yate's correction for discontinuity (Campbell, 1974) as indicated in the text. The level of significance is taken as $P < 0.05$ throughout unless stated otherwise.

RESULTS

Effects of Testosterone-Filled PDS Implants on the Male Reproductive System

The effects of different testosterone-filled PDS implant lengths on the male reproductive system of rats are shown in Tables 1 and 2. The objective of using these different treatments was to obtain animals with varying numbers of spermatozoa in the epididymis. To validate this experimental approach, a number of essential parameters are presented. The mean initial body weights for each of the seven groups of animals were not significantly different (Table 1). All groups of animals gained weight during the 110-day experimental period. The only group that had a significantly different mean final weight from any of the others was that treated with 8.0-cm implants.

There was no significant change ($P > 0.05$, Duncan's multiple range test) in serum testosterone in animals receiving implants from 0.5 to 8.0 cm in length. Seminal vesicle weights were not significantly changed with all doses of testosterone used with the exception of the 2.0- and 8.0-cm implants. The small but significant increase found in the seminal vesicle weights of the animals receiving 2.0-cm implants is surprising since the seminal vesicle weights of the two groups bracketing it are not different from control. The large and significant increase in seminal vesicle weight with the 8.0-cm im-

TABLE 1. The effect of testosterone-filled (T) PDS implants on body weights, seminal vesicle weights, serum testosterone and testes weights.

Treatment group	Body weight (g)		Serum testosterone (ng/ml)	Seminal vesicle weight (g)	Testes weight (g)
	Initial	Final			
Control	308 ± 8 ^a	562 ± 14	2.5 ± 0.3	0.69 ± 0.04	3.17 ± 0.07
0.5 cm T	303 ± 6	562 ± 14	3.2 ± 1.2	0.69 ± 0.04	3.29 ± 0.14
1.0 cm T	310 ± 6	569 ± 16	2.1 ± 0.4	0.66 ± 0.04	3.22 ± 0.13
2.0 cm T	305 ± 4	577 ± 15	1.4 ± 0.1	0.82 ± 0.05*	3.02 ± 0.08
3.0 cm T	334 ± 19	578 ± 21	1.2 ± 0.1	0.76 ± 0.06	1.63 ± 0.18*
4.0 cm T	323 ± 9	556 ± 24	1.8 ± 0.2	0.79 ± 0.04	1.48 ± 0.02*
8.0 cm T	323 ± 9	502 ± 12*	4.1 ± 0.8	0.97 ± 0.04*	2.11 ± 0.09*

^aValues represent means ± SEM (n=6).

*P<0.05, ANOVA and Duncan's multiple range test.

plant was expected since at this dose serum testosterone is higher than control.

Predictably, there was a biphasic change in testicular weight. Implant lengths up to 4.0 cm had either no effect or caused a decrease in testis weight. This decrease was significant in groups receiving 3.0-cm and 4.0-cm implants. It is interesting to note that there is an abrupt, nearly 50%, decrease in testes weights between groups of animals receiving 2.0-cm and 3.0-cm implants. The group receiving 8.0-cm implants also had mean testicular weights significantly lower than control. The weight was, however, nearly 50% greater than that of the 4.0-cm implant-treated animals ($P<0.05$).

The dose-dependent changes in epididymal head-body and tail weights (Table 2) are qualitatively similar to those found for testicular weights. The number of spermatozoa in the head-body and tail of the epididymis changed much more dramatically, however. Animals having means of 3%, 13% and 30% of the control head-body spermatozoal content were obtained in the 4.0-, 3.0- and 8.0-cm implant groups, respectively. Similarly, animals having means of 4%, 20% and 50% of the epididymal tail spermatozoal content were obtained in the 4.0-, 3.0- and 8.0-cm implant groups. Thus, the experimental design did result in animals having different decreases in sperm reserves.

Effects of Testosterone-Filled PDS Implants on Fertility

The occurrence of mating between each male and the two females in the cage was assessed by counting the number of seminal plugs in the tray beneath the cage on the morning following exposure (Fig. 1A). The mean numbers of plugs in all treatments were surprisingly similar, ranging between 3.8 and 6.0. There was only one treatment group, with 1.0-cm testosterone-filled PDS implants, that had a significantly lower number of plugs than control. This difference was, however, barely significant at the $P<0.05$ level. The value was not significantly different than that found in a number of other treatment groups, e.g., 3.0- and 4.0-cm implants.

The percent of sperm-positive females per number exposed, i.e., 24 per treatment group, remained within the 75 to 96% range for all groups with the exception of that treated with the 4.0-cm testosterone-filled PDS implants where the percent of sperm-positive females was reduced to 50% (Fig. 1B). It is interesting to note that an 80% reduction in the spermato-

TABLE 2. The effect of testosterone (T)-filled PDS implants on epididymal weights and sperm counts.

Treatment group	Epididymis			
	Head-body		Tail	
	Weight (g)	Sperm count ($\times 10^6$)	Weight (g)	Sperm count ($\times 10^6$)
Control	0.60 ± 0.01^a	103.7 ± 6.0	0.66 ± 0.01	174.5 ± 22.9
0.5 cm T	0.65 ± 0.03	113.2 ± 6.6	0.65 ± 0.02	169.6 ± 20.4
1.0 cm T	0.60 ± 0.03	109.0 ± 6.8	0.65 ± 0.03	223.9 ± 22.8
2.0 cm T	0.60 ± 0.01	101.7 ± 5.0	0.62 ± 0.03	172.3 ± 23.0
3.0 cm T	$0.38 \pm 0.03^*$	$13.1 \pm 11.0^*$	$0.33 \pm 0.04^*$	$34.5 \pm 32.3^*$
4.0 cm T	$0.39 \pm 0.02^*$	$3.1 \pm 0.6^*$	$0.30 \pm 0.02^*$	$7.0 \pm 2.8^*$
8.0 cm T	$0.45 \pm 0.02^*$	$31.0 \pm 7.1^*$	$0.48 \pm 0.03^*$	$88.7 \pm 14.9^*$

^aValues represent means \pm SEM (n=6).

*P<0.05, ANOVA and Duncan's multiple range test.

zoal reserves of the tail of the epididymis (Table 2) has no effect on the number of sperm-positive females per number exposed. The percent of pregnant females per sperm-positive females,

however, was significantly decreased in two treatment groups, i.e., those animals receiving 3.0- and 4.0-cm implants (Fig. 1C). In comparing Panels A, B and C of Fig. 1, it becomes evident that though testosterone treatment has no major effect on male sexual behavior there is a marginal decrease in the number of sperm-positive females and a major decrease in the number of pregnant females per sperm-positive females.

The mean number of spermatozoa in the tail of the epididymis (Table 2) and the total number of pregnant females per sperm-positive females (Fig. 1C) would suggest that these two parameters are proportional. However, careful examination of the data for individual males demonstrates that, in fact, there is an apparent minimal spermatozoal number in the cauda epididymidis necessary to obtain fertility and that over a wide range of caudal epididymal spermatozoal counts there is a similar rate of fertilization. In Fig. 2 the individual epididymal tail spermatozoal counts for all 42 males in the seven treatment groups are graphed. The proportion of each bar that is *batched* represents the percentage of the four exposed females that

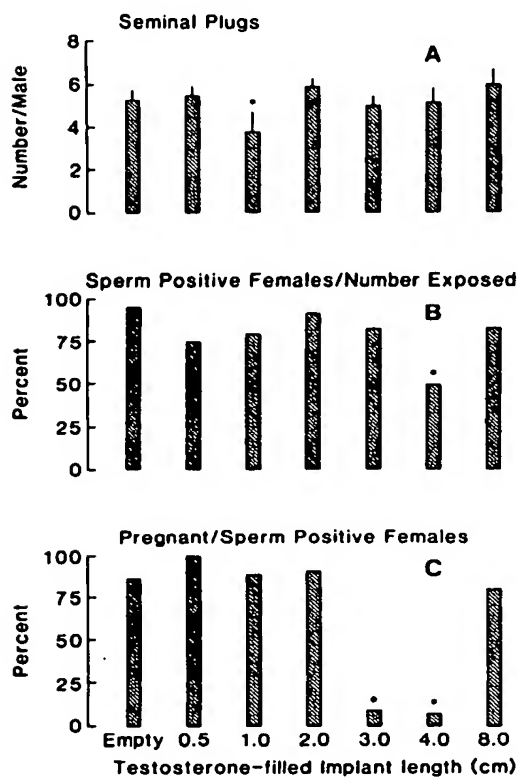


FIG. 1. Effect of testosterone-filled PDS capsules implanted subcutaneously for 3 mo in adult male rats (n=6 per group) on: A) Number of seminal plugs/male after overnight exposure twice to two females in proestrus. Values represent means \pm SEM. B) Percent of sperm-positive females/number of exposed females (n=24 per group). C) Percent of pregnant females/sperm-positive females. Starred values represent a statistically significant difference from empty implants (P<0.05) by Duncan's multiple range test (A) or chi-square analysis (B and C).

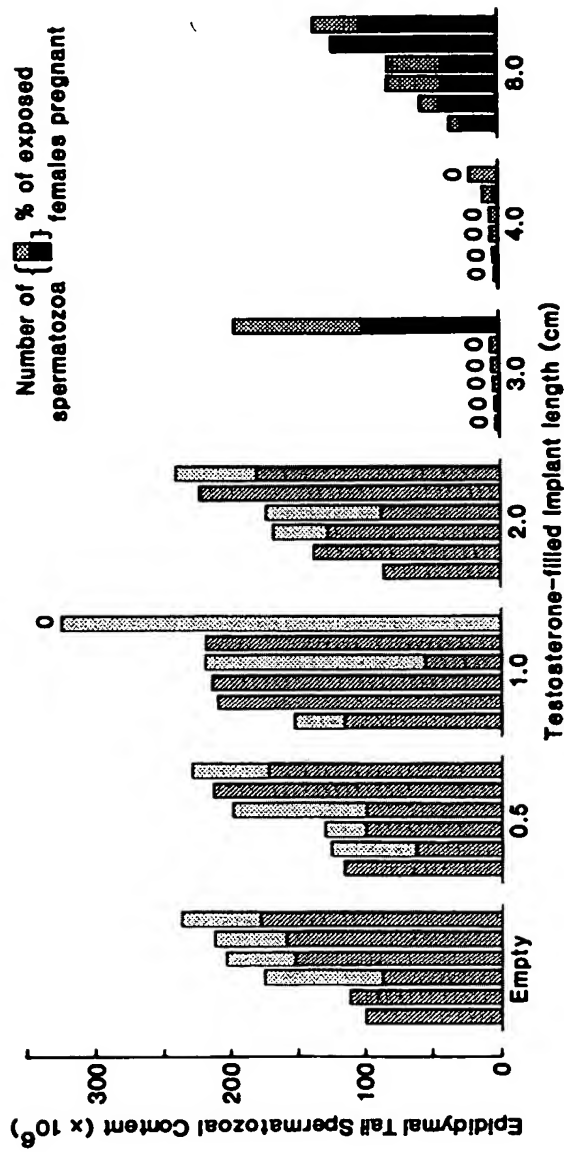


FIG. 2. Effect of testosterone-filled PDS capsules implanted subcutaneously for 3 mo in adult male rats (n=6 per group) on the number of spermatozoa in epididymal tail segments. The percent of exposed females (n=4 per male) that became pregnant is indicated by the fraction of each bar that is hatched. Zeros indicate that none of the exposed females were pregnant.

became pregnant. Four major points may be highlighted from this figure. First, no male whose epididymal sperm reserve was five million or less caused a female to become pregnant. Second, in the 3.0-cm implant group one male is responsible for the two pregnancies and contributes over 95% to the sum of the six animals with respect to epididymal tail spermatozoa; this "spurious" animal did not significantly differ from any of the other animals in this group with respect to the other parameters tested, e.g., serum testosterone or seminal vesicle weight. Third, epididymal tail spermatozoal reserves that ranged from about 30 to 200 million all resulted in at least one and usually two to four pregnancies out of four exposed females. Finally, it may be worth noting that the male (last animal in the 1.0-cm implant group) having an epididymal tail spermatozoal

content over 80 million greater than any other male did not impregnate any of the exposed females.

Effect of Testosterone-Filled PDS Implants on Pregnancy Outcome

To assess potential effects on fertilization and subsequent implantation, the numbers of corpora lutea and implantation sites were quantified (Fig. 3A). Since the females were not treated, the number of corpora lutea in all the groups was expected to be similar, which is indeed the case. The number of implantation sites in the 0.5-, 1.0-, 2.0- and 8.0-cm implant groups did not differ significantly from the control. Of the three out of 48 females that became pregnant after exposure to males bearing 3.0- and 4.0-cm implants, two had a

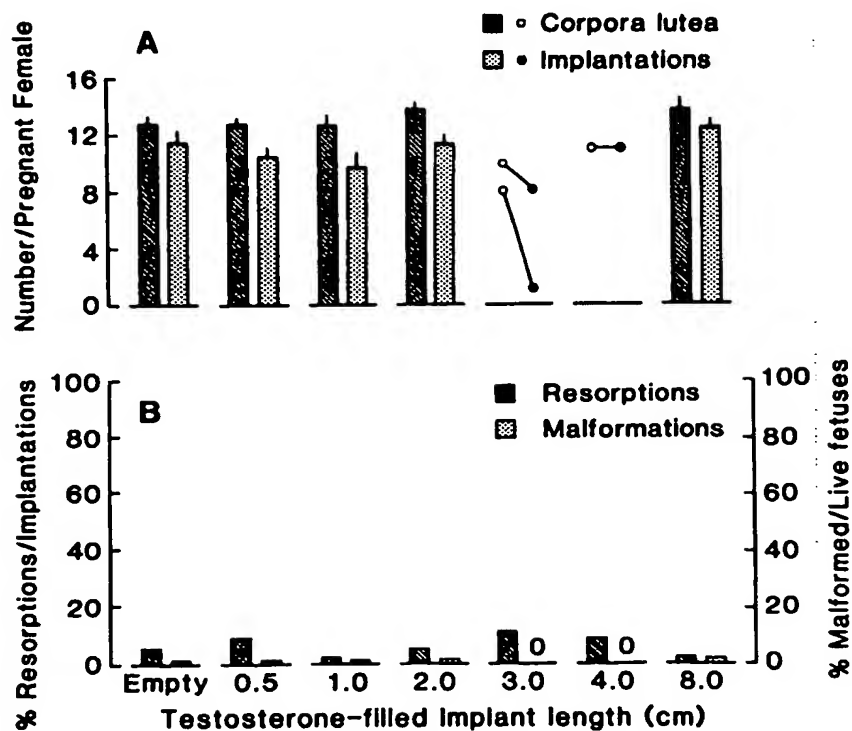


FIG. 3. A) The effect of testosterone-filled PDS capsules implanted subcutaneously for 3 mo in adult male rats (n=6 per group). A) The number of corpora lutea and implantations per pregnant female. Bars represent means \pm SEM. For the 3.0-cm and 4.0-cm implant groups individual values are presented. B) The percent of resorptions/implantations and percent of malformations/live fetuses. Zeros indicate an absence of observed malformations. No significant differences were found.

number of implantations (8 and 11) that were within the range of the control and other treatment groups. The third female, however, had only one implantation site. Thus, there was no marked effect of testosterone suppression of spermatogenesis on the number of implantation sites. This indicates that this treatment is not selectively deleterious to fertilization or early embryonic development.

Postimplantation embryonic death or resorptions and malformations for litters from control and treated males are shown in Fig. 3B. The rate of resorptions in all seven groups of animals ranged from 2 to 11% with the control value being 5.5%. No significant differences were found among these groups by chi-square analysis. The incidence of malformations among the live fetuses examined on Day 20 of gestation varied between 0 and 1% in all treatment groups (Fig. 3B). Though the two treatment groups with no malformed fetuses were the 3.0- and 4.0-cm implant groups, it would be inaccurate to assign a protective role to this treatment because the denominator is very low. Further, the types of malformations that were found in the progeny from animals treated with empty, 0.5-, 1.0-, 2.0- or 8.0-cm testosterone-filled implants were varied (Table 3). This spectrum of malformations is consistent with spontaneous malformations and not with any selective teratogenic effect of paternal treatment with testosterone.

The mean number of pups/litter was in the range of 9.6 to 12.1 for the control and all treatment groups. The only exception was the 3.0-cm implant group where the one male who fathered both litters (1 and 7 pups) had high epididymal spermatozoal reserves. The number of male and female pups in each litter is shown in Fig. 4A. It is clear from this figure that

testosterone treatment of the males and thus different levels of spermatogenesis does not result in a selective preference for one sex over the other. Further, the mean per litter weights of the male and female pups in the control and all treatment groups were very similar (Fig. 4B). The mean per litter weights of the male pups were consistently, but not significantly, higher than those of the female pups. It is interesting to note that the one surviving fetus (male) in one of the two litters from the male treated with a 3.0-cm implant had a weight that was nearly identical to the mean per litter weight of male pups in the control group.

DISCUSSION

The results presented above indicate that decreases in epididymal tail spermatozoal reserves do not result in proportional decreases in the number of progeny per litter. Rather, there can be substantial decreases (greater than 90%) in epididymal tail spermatozoal reserves without any effect on the fertilizing potential of the male or on the number of the resulting progeny. It is also evident that epididymal tail spermatozoal reserves that are 5 million or less are apparently not associated with any fertility.

All of the data presented above on the effects of administration of testosterone via subcutaneous release implants on the male reproductive system and, specifically, the biphasic effect on spermatogenesis, are consistent with previous reports in the literature (Berndtson et al., 1974; Reddy and Prasad, 1973; Robaire et al., 1979; Walsh and Swerdloff, 1973). The decrease in spermatogenic activity with low doses of testosterone is due to a major suppression of gonadotropins and consequently a decrease in intratesticular testosterone. The par-

TABLE 3. Types of malformations that were found in the progeny from animals with empty, 0.5-, 1.0-, 2.0- or 8.0-cm testosterone (T)-filled implants.

Treatment group	Number of malformed fetuses/live fetuses	Malformations
Control	1/221	Reduced tail diameter and length
0.5 cm T	1/173	Hydrocephaly, open left eye
1.0 cm T	2/140	Cleft mandible, omphalocele
2.0 cm T	2/203	Reduced tail diameter and length, omphalocele
3.0 cm T	0/9	---
4.0 cm T	0/10	---
8.0 cm T	1/189	Microphthalmia, micrognathia

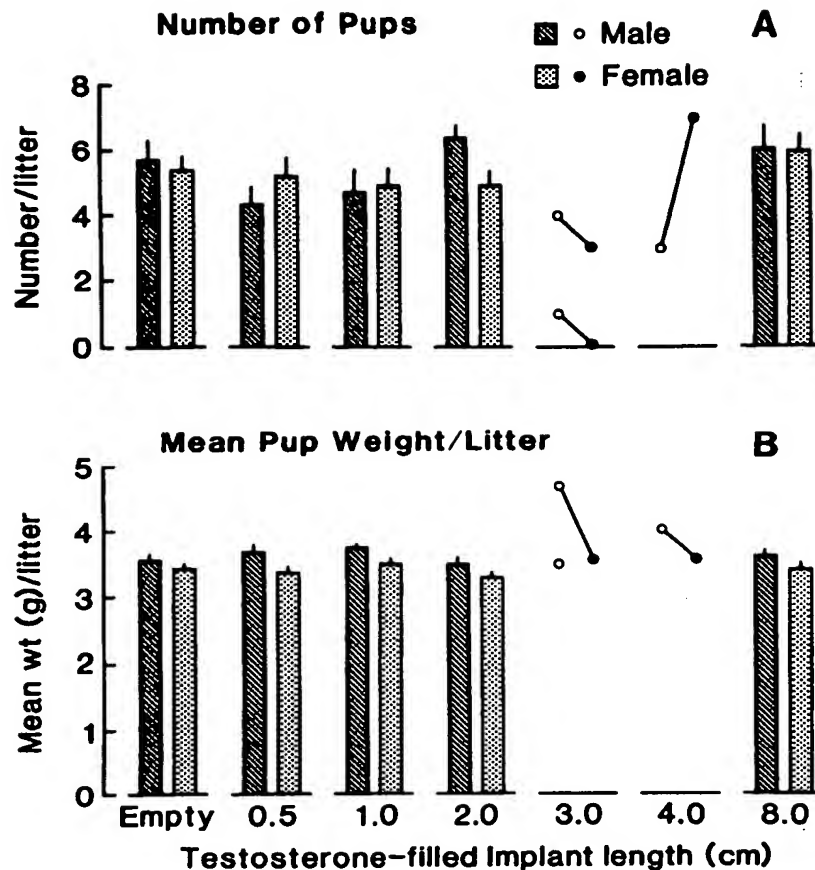


FIG. 4. Effect of testosterone-filled PDS capsules implanted subcutaneously for 3 mo in adult male rats ($n=6$ per group). A) The mean number of male and female pups/litter. B) The mean weights of male and female pups/litter. Bars represent means \pm SEM. For the 3.0- and 4.0-cm implant groups individual litters are indicated.

tial or complete maintenance of spermatogenesis with high doses of testosterone is associated with large increases in serum testosterone; it is presumably this high circulating level of androgens that can account for the maintenance of spermatogenesis. The ability of high doses of testosterone alone to maintain spermatogenesis in hypophysectomized animals is further evidence of the above presumption (Robaire and Zirkin, 1981).

There is a dearth of studies using animal models where the issue of the relationship between the number of spermatozoa in the male reproductive tract and fertilizing potential of the male is addressed. There are, however, a

number of relevant clinical studies in man where attempts have been made to correlate spermatozoal numbers in the ejaculate with fertility (Smith and Steinberger, 1977; Bostofte et al., 1982). Though these studies have a number of inherent limitations, the data indicate that men having 10 million spermatozoa per ml or less in the semen can have progeny; the percentage of fertile men in this group, however, is far inferior to that of groups of men having more than 20 million spermatozoa per ml. Because of the continuous fluctuations in seminal concentrations of spermatozoa (Belsey et al., 1980), there is still some doubt as to the ability of men having less than 5 million sper-

matozoa per ml to father children. The study of Barfield et al. (1979) would indicate that in men whose spermatozoal concentrations have been driven down by administration of a contraceptive (i.e., medroxyprogesterone acetate and testosterone esters), fertility may result even with spermatozoal concentrations less than 1 million/ml; thus, this raises the question of whether a low spermatozoal count obtained as a result of contraceptive treatment has a higher "fertilizing potential" than a similar spermatozoal count in a nontreated man. Most clinical studies, because of the problem of collecting serial semen samples, cannot adequately assess spermatozoal reserves. Furthermore, obvious ethical reasons prevent clinical assessment of a contraceptive with respect to its ability to induce abnormal progeny.

In a study of over a thousand control patients, Bostofte et al. (1982) indicated that the percent of pathological pregnancies in men having 5 million or less spermatozoa per ml of semen was not significantly different from that of men having higher seminal spermatozoa concentrations.

The concern that decreased spermatozoal production associated with contraceptive treatment may somehow select a population of spermatozoa that will produce a higher incidence of abnormal progeny was addressed here. The present study demonstrates that decreasing spermatozoal reserves with sustained testosterone administration still results only in normal progeny. Furthermore, there was no increase in either pre- or post-implantation loss. These results support the observation reported by Ewing et al. (1979) that 2.5- and 4.0-cm testosterone-filled implants given to male rats did not increase postimplantation loss.

Whether the use of other potential contraceptive agents such as estrogens, progestagens and LHRH analogs in conjunction with testosterone can result in increased embryonic or fetal mortality or abnormal progeny remains to be elucidated. However, it is clear from the present study that the reduction in spermatogenesis mediated by testosterone alone does not produce these deleterious effects on pregnancy outcome.

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Perspective

Intracellular Receptors and Signal Transducers and Activators of Transcription Superfamilies: Novel Targets for Small-Molecule Drug Discovery

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Introduction

The proliferation and differentiation of mammalian cells is modulated by a number of specific signal molecules that regulate gene expression. Among these signals are (i) the steroid hormones (*e.g.*, glucocorticoids, mineralocorticoids, estrogens, progestins, and androgens), chemical messengers produced by the body in response to a variety of stimuli; (ii) small-molecule hormones including thyroid hormone, calcitriol (a vitamin D₃ metabolite), and the retinoids; and (iii) the cytokine superfamily of protein molecules that affect cells of the immune and other systems.

The mechanism of action of steroid hormones has been studied extensively over the last 25 years. The steroid hormones, which have similar fused ring skeletons, vary structurally with the placement of double bonds and the nature of their side chains. Dramatic differences in biological activity of these molecules are due to interactions with specific receptors capable of distinguishing minor differences in chemical structure. In contrast, nonsteroidal small-molecule hormones vary significantly in chemical structure; however, they utilize similar receptors to produce their biological effects. The receptors for the nonsteroidal small-molecule hormones include the retinoic acid (RA) receptor subtypes (RAR α , RAR β , RAR γ , RXR α , RXR β , and RXR γ), the vitamin D receptor, and the thyroid hormone receptor subtypes (T₃R α and T₃R β and splice variants).

The definition of biochemical events that mediate signal transduction in response to steroid hormones and small-molecule hormones has advanced rapidly, beginning in 1985 with the first cloning of an intracellular receptor (IR).¹ Molecular biological techniques enabled subsequent cloning and characterization of receptors for

each of the steroid and small-molecule hormones. This dramatically enhanced understanding of hormone action and led to a number of unifying insights concerning the receptors and their ligands. The IRs are closely related members of a protein superfamily² that have apparently diverged from a common ancestral gene.³ The presence of a specific IR within a cell enables that cell to respond to the hormone cognate to that IR. The IRs share a common mechanism of action, since they in general remain latent inside target cells until exposed to their specific ligands, which activate them as transcription factors producing specific changes in gene expression.

Cytokines are a large and diverse family of circulating polypeptides produced by many different cell types. They include various types of interferons (*e.g.*, IFN- α , - β , - γ), the interleukins (*e.g.*, IL-6), the colony-stimulating factors (*e.g.*, granulocyte colony-stimulating factor, G-CSF), and growth factors (*e.g.*, epidermal growth factor, EGF). Individual cytokines act upon a variety of cell types. As polypeptides, cytokines cannot freely enter cells; they act by binding to specific cell surface receptors. The understanding of the biochemical events by which some of the cytokines achieve their distinctive biological effects has increased significantly since 1992. There is a surprising degree of underlying similarity in the pathways of cytokine signal transduction, explained by the discovery of a family of latent cytosolic proteins, termed signal transducers and activators of transcription (STATs),⁴ that mediate signal transduction for the majority of the cytokines. This newly defined STAT protein superfamily acts to mediate specific changes in gene expression and consequently cell function following exposure to most cytokines.

Both the IRs and the STATs act as DNA-sequence-

specific transcription regulators, selecting the genes expressed and modulating the level of their expression within a cell following exposure to a specific stimulus. The DNA sequence-specific factors that act to modulate gene transcription are collectively termed transcription factors and include the IRs and STATs, which exert their effects by binding to chromosomal DNA in a sequence-specific manner or by interacting with components of the transcription apparatus, the complex of RNA polymerases and accessory proteins that carry out the production of messenger RNA (mRNA). Transcription factors control the expression of specific sets of genes. Since the pattern of gene expression determines cell function, the control of gene expression is a central process in biology.

The concepts and terminology used to describe the transcription of eukaryotic genes will be briefly reviewed here, preceding a detailed discussion of the mechanics of the IR and STAT signal transduction pathways and their relevance to drug discovery. Transcription, the rate-limiting step in gene expression, is used by the cell as a primary point of regulation for subsequent events controlled by hormones and cytokines. The regulatory pathways involved in transcription are controlled by protein-protein and protein-DNA interactions.

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals is a complex process involving the concerted action of many proteins. RNA polymerase II is the enzyme responsible for the production of mRNA from genes in eukaryotes, as outlined in Figure 1.⁶ It acts in combination with a number of transcription factors. These factors can be divided into three classes on the basis of their functions. First are the basal transcription factors, which are required for an unregulated, basal level of transcription by RNA polymerase II. Second are the DNA-sequence-specific transcription factors, which are required for regulated transcription of a subset of these genes. Lastly, the coactivators represent a newly discovered class of regulatory proteins that act in concert with sequence-specific and basal transcription factors to further modulate levels of transcription.

The regulatory region in the immediate vicinity of the transcription start site is termed the promoter and contains a number of core response elements. Response elements are specific nucleotide sequences that are recognized by and act as binding sites for transcription factors. The core response elements are usually located within several hundred base pairs of the transcription start site. The most common core response element among genes transcribed by RNA polymerase II is the 5'-TATAAA-3' sequence (TATA box), recognized by a specific basal transcription factor. Other response elements commonly found in the promoter region and to which specific transcription factors bind include the 5'-GGGCG-3' and 5'-CCAAT-3' sequences. In addition, mammalian genes may contain particular combinations of positive or negative regulatory response elements that are uniquely arranged as to number, type, and spatial organization. These response elements are the binding sites for sequence-specific transcription factors that activate or repress gene expression for the gene downstream from that promoter. A response element that regulates the activity of the promoter from a distance and in an orientation-independent fashion is termed an

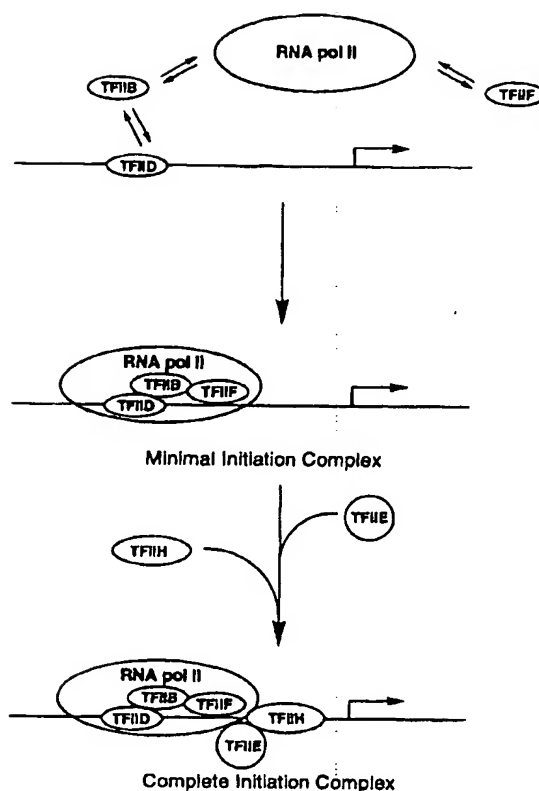


Figure 1. The cascade of events leading to transcriptional initiation. The first step in the initiation process requires binding of TFIID to the TATA box of the promoter. After TFIIB binds to TFIID on the DNA, RNA polymerase II and TFIIF are recruited to the complex of proteins at the transcription initiation start site. Transcriptional initiation also requires the presence of TFIIE and TFIIH to yield a complete initiation complex.

enhancer or a silencer depending on whether it induces or represses gene expression. Both the IRs and STATs interact with response elements to control transcription.

Initiation of transcription requires that the basal transcription factors, by interaction with core response elements, form an initiation complex, the active assemblage of RNA polymerase II and accessory proteins required to start RNA synthesis. A schematic representation of initiation complex formation is shown in Figure 1. The first step in the assembly of the initiation complex is the binding of the transcription factor D for RNA polymerase II (TFIID)⁶ to the TATA box. TFIID is a multiprotein complex that consists of the TATA binding protein (TBP) and TBP associated factors. TFIID acts as a binding site for TFIIB. Once bound, TFIIB is able to recruit RNA polymerase II and TFIIF to the transcription start site. The complex formed by the association of these factors is stable; however, subsequent association of transcription factors TFIIE and TFIIH is required to complete formation of the transcription initiation complex to begin mRNA production.^{5,7}

Sequence-specific transcription factors modulate the formation of the initiation complex and thus control the frequency of transcription of a specific subset of genes

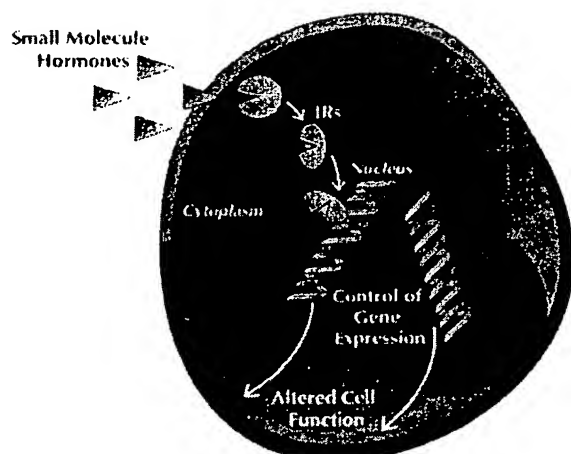


Figure 2. Control of gene expression through the intracellular receptor signaling pathway. In the IR pathway, small-molecule ligands diffuse into the cell and bind to the appropriate receptor. This leads to a conformational change of the receptor that causes dissociation with proteins such as hsp90 that are associated with the inactive form of the receptor and allows receptor binding to a specific response element in the promoter of a gene controlled by that hormone. Binding of an activated IR protein to its response element modulates the transcription of the downstream gene and, thus, translation of the gene product.

by RNA polymerase II. These transcription factors may act (i) by binding to sequence-specific response elements (enhancers or silencers) within the DNA or (ii) *via* direct protein-protein interaction with basal transcription factors within the initiation complex.

Recently, coactivators have been identified as a third class of transcription factors. It has been proposed that these function as physical links between the sequence-specific transcription factors and one or more components of the initiation complex. The TFIID complex, for example, contains several proteins that are tightly associated with the TBP and display some coactivator function. These proteins may be unnecessary for basal level transcription but essential for stimulation of transcription by sequence-specific transcription factors.

The remainder of this review will focus on two families of sequence-specific transcription factors: (i) the IRs, the transcription factors through which steroid hormones and small-molecule hormones control gene expression, and (ii) the STATs, the transcription factors through which many cytokines and growth factors control gene expression.

Information regarding hormonal and cytokine signal transduction that has delineated the underlying principles, properties, and biological roles of IRs and STATs will be discussed. Important implications for new drug discovery approaches, targets, and tools with the potential to yield breakthrough small-molecule drugs mimicking or blocking hormone and cytokine actions will be highlighted.

Intracellular Receptor Function

The sequence of events involved in IR signal transduction is shown in Figure 2. Briefly, the non-peptide hormones, such as estrogen or RA, are sufficiently lipophilic to diffuse freely through the cell membrane

without the need for specialized transport systems. A number of these hormones interact with plasma and intracellular binding proteins that show varying degrees of specificity; however, the actual mediators of hormonal signal transduction are the IRs. The intracellular receptors, proteins with molecular masses that range from approximately 55 to 90 kDa, have a characteristic domain structure. They are located within the cell, but are not cell membrane associated. Once a hormone enters the cell, it binds to its IR with high affinity, resulting in a conformational change in the receptor, activating the IR as a transcription factor.

In the absence of bound ligand, inactive steroid hormone receptors are sequestered in cells in a complex with the heat shock proteins hsp-90, hsp-70, and p59.⁸ Additional proteins, such as YDJ1, also appear to influence the activation of steroid hormone receptors.⁹ The cellular localization of the unliganded complex (cytoplasmic or nuclear) remains controversial.¹⁰ The conformational change that occurs in the receptor as a consequence of hormone binding results in the dissociation of the IR from the heat shock proteins and release of the monomeric receptor molecule and its ligand from the complex. In contrast to the steroid hormone receptors, the inactive small-molecule hormone receptors do not appear to interact with heat shock proteins and, in the absence of hormone, are located in the nucleus.¹¹ The binding of hormone also results in conformational changes in these receptors and in their subsequent activation. The exact nature of these conformational changes is not known. However, for some of the IRs it can be shown to involve alteration in the accessibility of the IR's C-terminal region, detectable either with immunological reagents¹² or by determination of ligand modulation of proteolytic susceptibility *in vitro*.¹³

There are approximately 10000 genes expressed in all cells, with 10000–20000 expressed in a single cell. Of these, only a few hundred genes in any cell are regulated by IRs. Ligand-activated IRs exert their effects by binding directly to specific chromosomal enhancer sequences termed hormone response elements (HREs) that are located within the regulatory regions of target genes.^{14,15} Once bound to HREs, the activated receptor increases the transcriptional activity of the adjacent promoter, resulting in optimal expression of the target gene. Each HRE is made up of two approximately hexanucleotide half-sites separated by a variable number of nucleotides. The sequence of the half-sites and the number, but not the sequence, of the spacing nucleotides are key determinants of the specificity of IR interaction. HREs differ in their nucleotide sequences as well as the orientation and spacing of their half sites. Comparison of the sequences of the HREs from different hormone responsive genes indicates that a similar motif is used by each of the receptor subfamilies (Table 1).

Recent evidence shows that the IRs can associate to form homodimers, heterodimers, and possibly other oligomeric receptor species.^{16,17} These dimers may bind to inverted repeats, direct repeats, or everted repeats. It is generally believed, in the case of the glucocorticoid receptor (GR),¹ estrogen receptor (ER),¹⁸ progesterone receptor (PR),^{19,20} androgen receptor (AR),^{21–23} and mineralocorticoid receptor (MR)²⁴ that the active receptor species are homodimers. For many of the other IRs, including thyroid hormone receptor (TR),²⁵ RA receptors

Table 1. Hormone Response Elements

Receptor	Example of HRE	Consensus Sequence
GR, MR	MMTV GTTACA AAC TGTCT	GGTACANNN TGTCT
AR & PR	TO TGCACA GCG AGTCT	-
	TAT TGTACA GGA TGTCT	-
ER	cvt GGTCA GCG TGACC	GGTCANNN TG ^A CC
	rPl TGTCA CTA TGTCC	-
RXR	rCRBP II AGGTCA C AGGTCA	AGGTCA N AGGTCA
VDR	hOST GGGTGA ACG GGGGCA	AGGTCAANN AGGTCA
TR	NMHC AGGTGA CAGG AGGACA	AGGTCAANNN AGGTCA
RAR	hRAR β GGTCA CCGAA AGTCA	AGGTCAANNNN AGGTCA

MMTV, mouse mammary tumor virus; TO, tyrosine oxidase; TAT, tyrosine aminotransferase; cvt, chicken vesicular stomatitis; rPl, rat prolactin; rCRBP II, rat cellular retinoic acid-binding protein type II; hOST, human osteocalcin; NMHC, human cardiac myosin heavy chain; hRAR β , human RAR β ; -, inverted repeat of half-site; ~, direct repeat of half-site.

(RARs),^{26,27} vitamin D receptor (VDR),²⁸ and a number of the orphan receptors, the functional transcription factor is a heterodimer formed with a member of the retinoid X receptor (RXR)^{29,30} subfamily.

The idea that the receptors can only bind to HREs as dimers is being reexamined since the estrogen receptor appears able to bind as a monomer to a single half-site of either the estrogen response element or the thyroid hormone response element.^{31,32} Further, two recently described proteins with significant primary sequence homology to members of the IR superfamily, nerve growth factor I-B (NGFI-B)^{33,34} and steroidogenic factor-1 (SF-1),³⁵ also bind to half-sites as monomers. In each case, the HRE is an extended estrogen response element with extra 5' nucleotides. Although not clearly understood, the selection of the DNA-binding mode appears to be determined by response element type, promoter context, and relative levels of the pertinent IRs.

Intracellular Receptor Structure

The cloning and sequencing of cDNAs for the IRs and comparison of their deduced amino acid sequences show that the superfamily members are modular in structure.^{2,36} Sequence data and functional analysis show the IRs to consist of six discrete subdomains, A-F (Figure 3). Three of these domains have been described in detail: (i) the DNA-binding domain, which is highly conserved and provides specific binding to the HRE, (ii) the ligand-binding domain located C-terminal to the DNA-binding domain, which provides a hydrophobic pocket for the binding of the ligand but also contains a number of other functionally important regions, and (iii) the C- and N-terminal transactivation domains, which are more variable in sequence.

I. DNA-Binding Domain. The DNA-binding domain, which is usually centrally located in the primary sequence of an IR, is composed of 68 amino acid residues. Of those 68 amino acids, 20 are invariant and determine the generalized DNA-binding structure.³⁷ Confirmation that this region is the DNA-binding domain was obtained using XFACS,³⁸ 2D NMR,^{39,40} and X-ray crystallography.⁴¹ The DNA-binding domain is

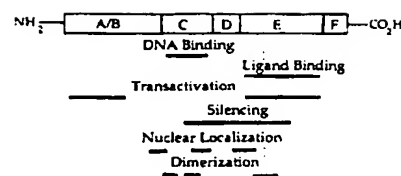


Figure 3. The domain structure of the intracellular receptors. The DNA-binding domain (DBD) corresponds to the C region and the ligand-binding domain (LBD) corresponds to the E region. These critical domains are restricted to defined segments of the protein. However, the segments of the receptor responsible for transactivation, nuclear translocation, and dimerization are not restricted to single defined domains, but are found in multiple regions of the receptor. The region marked D is a short region known as the hinge region that connects the DBD and the LBD and has functional activity as shown in the figure. The DBD contains nine cysteine residues enabling the coordination of two zinc ions and the formation of two zinc fingers. The first zinc finger contains the P box responsible for the sequence specificity of the IR; the second zinc finger of the DBD contains the D box, which discriminates between similar response element sequences with different spacing between the half-sites of the response element.

highly basic and contains nine cysteine amino acid residues. The presence of the cysteine residues enables the coordination of two zinc ions and the formation of two "zinc fingers". Selection of the specific HRE to which the receptor binds is determined by the three amino acids at the base of the first finger.¹⁵ Mutational analysis of the estrogen receptor showed that conversion of these three amino acid residues to the corresponding residues from the glucocorticoid receptor results in a mutant estrogen receptor that binds the glucocorticoid response element.⁴² The finger segment encompassing these residues, responsible for sequence specificity or selectivity of DNA binding, is termed the Proximal or P box.^{43,44} The sequence spanning cysteine residues 5 and 6, in the second zinc finger, is responsible for discriminating between response elements with similar sequences but different half-site spacing. This sequence of five amino acid residues is called the Distal or D box.⁴⁵ For example, the D box in the RXR recognizes a one nucleotide spacing between the response element half-sites (5'-AGGTCA n AGGTCA-3'), while the RAR D box recognizes half-sites with five spacing nucleotides (5'-AGGTCA nnnnn AGGTCA-3').

II. Ligand Binding Domain. The C-terminal or E region of the IRs is approximately 25 kDa and contains the ligand-binding domain (LBD), which determines the ligand-binding specificity of each receptor.^{2,36} Proteolytic mapping of the receptor indicated that a portion of the D region is necessary for binding of ligand with maximal affinity.¹² The hormonal ligands generally bind to their cognate IRs with affinity constants (K_d values) on the order of 1 nM. Upon binding hormone, the receptor is thought to undergo a major conformational change that results in its activation.¹² The crystal structure of the RXR α ligand binding domain has been reported.⁴⁶ However, since the crystal structure was determined in the absence of a ligand, the conformational changes induced by hormone binding have not yet been clearly defined. The ligand-binding domain additionally contains regions allowing (i) the dimerization of the receptor monomers,^{45,47,48} (ii) the interaction of selected IRs with heat shock proteins,⁸ (iii) nuclear translocation signals,⁴⁹ and (iv) one of

several transcriptional transactivation domains of the receptor. The dimerization⁵⁰ and nuclear translocation⁵¹ signals that have been mapped to the ligand binding domain are dependent upon the binding of hormone for their action. Nuclear translocation and dimerization signals are also present in the DNA-binding domain, but their action is hormone independent. Analysis of the region of the ligand binding domain that contains the dimerization signal has revealed a heptad repeat of hydrophobic residues that are highly conserved within the IR superfamily. This observation suggests that the nuclear hormone receptors dimerize via a leucine-zipper type mechanism.⁵²

III. Transactivation Domains. Transactivation domains are located in both the N-terminal and C-terminal regions of the receptors. Transactivation achieved with the DNA-binding domain alone represents a small portion of the total activity of the receptor. Deletion of the E regions of GR and ER, however, abolishes both hormone-binding and transactivation functions of the receptors. Since nuclear localization is a prerequisite for transactivation, it is difficult to assess the influence of the E region independent of its nuclear translocation function. In addition, at least in the case of the estrogen receptor, transactivation has been shown to be dependent upon the binding of hormone.⁵³ The situation is further complicated by the possibility that dimerization of the receptor may be necessary for efficient transactivation. However, careful mutational analysis, together with experiments involving chimeric genes, has enabled identification of specific regions that contain the transactivation functions for a number of the IRs. The transactivation domains, located N-terminal and C-terminal to the DNA-binding domain, have been termed transactivation unit 1 and 2, respectively (Tau 1 and Tau 2), in GR,⁵⁴ transactivation function 1 and 2 (TAF-1 and TAF-2) in ER,⁵⁵ and activation function 1 and 2 (AF-1 and AF-2) in PR.⁵⁶ The Tau 1, TAF-1, and AF-1 activation domains are located within the region marked A/B in Figure 3; the second activation domain of these receptors can generally be found in the LBD. The retinoid receptors, RARs and RXRs, have also been reported to have two domains responsible for transactivation, one each in the N-terminal and C-terminal regions.⁵⁷

Application of Intracellular Receptor Technology

Elucidation of the mechanism of IR-mediated transcriptional activation has enabled the development of high-throughput assays to detect novel small molecules that act as agonists, antagonists, or partial agonists of the IRs.⁵⁸ These assays can reveal the consequences of the interaction of any compound with any IR, using the cloned human IR cDNAs. These assays, termed cotransfection assays, are capable of detecting the functional effects on gene expression of small molecules that interact with specific IRs in mammalian cells. As shown in Figure 4, a gene for an IR that is a potential drug target is introduced by transfection (a procedure to

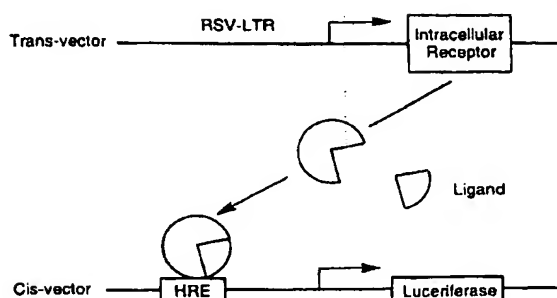


Figure 4. The cotransfection assay. Two plasmids are cotransfected into the appropriate cell background. One plasmid constitutively expresses an intracellular receptor. In the presence of ligand, the receptor acts in *trans* to bind to its HRE (the *cis* element) and activate transcription of the downstream reporter gene. The reporter is generally an easily detected enzyme such as chloramphenicol acetyl transferase (CAT) or luciferase.

induce cells to take up foreign DNA) into a mammalian cell lacking endogenous IRs of the type being studied. The introduced receptor cDNA directs the recipient cells to synthesize the receptor protein. A second gene under the transcriptional control of the appropriate HRE is also introduced by transfection (*i.e.*, cotransfected) into the same cells together with the IR gene. The protein product of this second gene functions as a reporter for the transcription-modulating activity of the receptor protein at its HRE. Thus, the reporter acts as a surrogate for the products normally expressed by genes under the control of the target receptor and its natural hormone. The reporter gene is chosen to encode a protein product that can be readily detected and quantified. Enzymes are useful reporters because they can often be assayed easily and, under the appropriate assay conditions, the rate of the reaction they catalyze directly reflects the amount of enzyme present. Firefly luciferase is an example of a frequently used reporter. The promoter that controls expression of the luciferase cDNA in the reporter plasmid is constructed to contain the appropriate HRE so that expression is under hormonal control at the level of transcription.

The cotransfection assay can be used to detect agonists for the target IR. A day after exposure to an appropriate agonist ligand in the medium bathing the transfected cells, an increase in reporter activity can be measured in cell extracts, reflecting ligand-dependent, IR-mediated increases in reporter gene transcription.

The cotransfection assay can also be used to detect the activity of small molecules that antagonize the activity of an agonist ligand for any IR. To detect antagonists the assay is carried out in the presence of a constant concentration of a known agonist sufficient to induce a constant reporter signal. Cells incubated in the presence of increasing concentrations of an antagonist will display progressive decreases in reporter signal. The cotransfection assay is therefore useful to detect both agonists and antagonists of specific IRs.

Current Therapeutics

Hormone-based therapies have been part of clinical medicine for over a century. Initially, extracts of endocrine glands were used as replacement therapies to supplement patients with glandular deficiencies.

Subsequently, the hormones themselves, purified from such extracts, were administered for similar uses. As the structures of the hormones were determined, chemically-synthesized versions replaced many of the naturally-derived hormone drugs. Subsequently, chemical analogues of these were synthesized and tested in animals to find compounds with improved therapeutic profiles relative to those of the hormones. Hormone agonists in clinical use include estrogens, anti-inflammatory glucocorticoids such as cortisone and dexamethasone, thyroxine, vitamin D₃ (a precursor to calcitriol), various progestins, and estrogens used in oral contraception, and vitamin A metabolites.

The sex steroids drive the growth and function of the tissues of the reproductive tract and breasts. Malignancies arising in these organs, such as breast or prostate cancer, derive from normal tissue and often are dependent on sex steroids to maintain growth. Initially, these cancers were treated by surgical ablation of the endocrine glands involved in the secretion of the sex steroids. The surgical removal of the glands that produce an "unwanted" hormone is drastic and far from satisfactory. It was therefore a great advance when hormone antagonist drugs first became available. These hormone antagonists were developed, as were the early hormone agonists, by a laborious process of chemical synthesis of drug candidates and testing in animals. Steroid hormone antagonists with clinical utility include the anti-estrogen tamoxifen, the anti-progestin mifepristone (RU486), and the anti-androgens flutamide and cyproterone acetate. Other clinically useful hormone antagonists include the anti-mineralocorticoid spironolactone.

Identification of Targets for the Discovery of Novel Drugs

The number of diseases that are associated with inappropriate production of or response to hormonal stimuli highlights the medical and biological importance of these effectors. The recent advances in our understanding of the molecular basis for the action of IRs offer the opportunity to improve many of the existing IR-modulating drugs. Despite the clinical utility of currently available hormone agonists and antagonists, many of the compounds are limited by their side-effect profiles. Delineation of the mechanism of a specific biological response enables the identification of small molecules that retain the efficacy of the IR-modulating drug, but have significantly improved side-effect profiles. Development of compounds more selective for the target IR and, thus, in the function(s) that they elicit are discussed in section I, below.

The discovery of receptor isoforms for a variety of receptor systems has already led to the development of more specific and improved drugs. These include the cardioselective β -adrenergic blockers and the receptor subtype selective antihistamines. For certain non-protein hormones, identification of intracellular receptor subtypes, many of which are expressed in a tissue specific manner, not only implies a specific physiological role for each subtype but may offer better defined pharmacological effects and thus potential for development of highly selective drugs. The utility of this approach for drug discovery is detailed in section II, below.

Another recent finding, offering additional potential for the discovery of new drugs, is the identification of several dozen "orphan" members of the IR superfamily. In most cases, ligands that activate these so-called orphan receptors have yet to be identified. These IRs may represent as yet uncharacterized signal transduction pathways for novel endocrine and paracrine systems or may include subtypes of IRs for known ligands. In either case the orphan IRs, discussed in section III, below, are of great interest for discovery of more selective and efficacious small molecule drugs.

I. Tissue-Selective Intracellular Receptor Activators. Progesterone is produced in the ovaries, testes, adrenal cortex, and placenta. Along with estrogens, progesterone is critical in preparing the female reproductive tract for reception of sperm and implantation of a fertilized ovum. Progestins and estrogens cause growth and development of the reproductive tract and breasts. Progesterone is responsible for the body temperature rise upon ovulation and is also critical for the maintenance of pregnancy.

Therapeutic uses for progestins may include contraception (in combination with estrogens) and control of dysfunctional uterine bleeding, dysmenorrhea (in combination with an estrogen), endometriosis, and threatened spontaneous abortion. The traditional steroidal agonists of the progesterone receptor (e.g., norgestrel and norethindrone) were synthesized over 25 years ago and exhibit anti-estrogenic activity along with varying degrees of cross reactivity with AR and GR.⁵⁹ While some cross reactivity with other receptors may occasionally be desirable,⁶⁰ opportunities now exist to prepare selective, easily synthesized progesterone receptor agonists and antagonists. Two functionally different approaches by which this may be achieved will now be discussed.

The recent discovery that the human progesterone receptor (hPR) exists in two forms, hPR-A (94 kDa) and hPR-B (120 kDa), opens one avenue for drug discovery. These receptors differ by 164 amino acids, which are present in the N-terminal region of hPR-B but absent from hPR-A. The two receptors have identical DNA and ligand binding domains and may⁶¹ or may not⁶² be present in equimolar concentrations in tissues. Hetero- and homodimers of hPR-A and hPR-B form upon ligand activation. Recent data suggest that the cellular pathways used by hPR-A and hPR-B are distinct.⁶³ The hPR-A has been demonstrated to inhibit the transcriptional activity of the glucocorticoid,⁶⁴ estrogen,⁶⁴ androgen, and mineralocorticoid⁶⁶ receptors in a cell- and promoter-specific manner. These properties may facilitate the identification of more selective compounds or compounds with cross reactivity by allowing *in vitro* analysis of progestin action. For example, hPR-A inhibition of ER may be a mechanism through which the anti-estrogenic properties of progestins are produced. These observations, if they apply *in vivo*, set the stage for discovering a new generation of hPR modulators.

Anti-progestins are currently used acutely as abortifacients. Other possible therapeutic uses may include cervical ripening and treatments for endometriosis, uterine fibroids, meningioma, and breast cancer. The anti-progestins are predominantly 11 β -aryl-19-nor steroids⁶⁷, (e.g., mifepristone, onapristone and Org 31806, Figure 5).⁶⁸⁻⁷⁰ Cross reactivity with the androgen, glucocorticoid, and estrogen receptors is a feature of

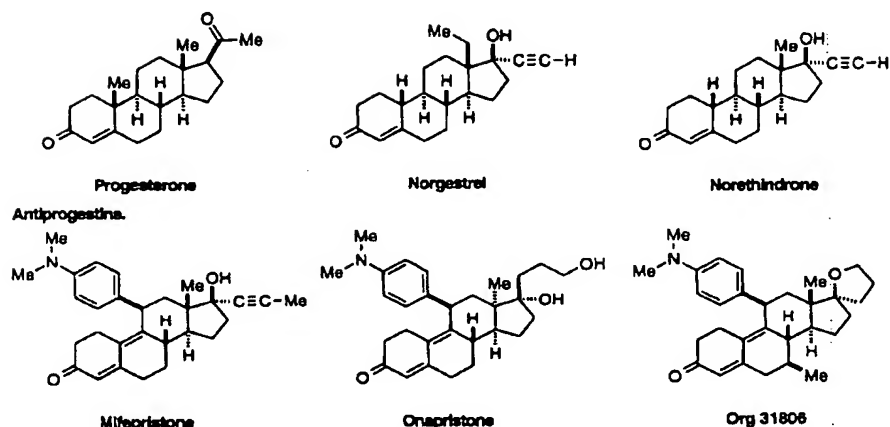


Figure 5. Progesterone-receptor agonists and antagonists.

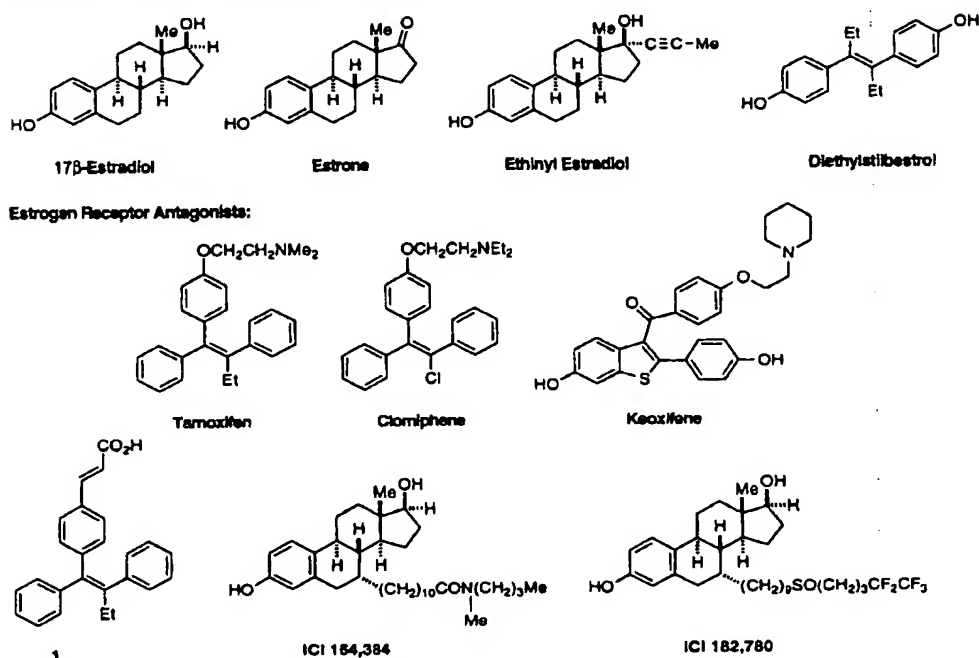


Figure 6. Estrogen receptor modulators.

current anti-progestins, which potentially limits their tolerability for chronic administration. Mifepristone⁶⁸ is a potent anti-progestin, anti-glucocorticoid, and anti-androgen that also exhibits anti-estrogenic behavior. These cross reactivities, while of little consequence for acute uses, may be detrimental in chronic therapy.

Substantial progress has been made to prepare more selective 11β-aryl steroidal anti-progestins; however, these compounds are difficult to synthesize.⁶⁷ Furthermore, some evidence suggests that there are two classes of anti-progestins among the 11β-aryl steroids⁷¹ that can be differentiated by the affinity of the ligand-bound dimerized receptor complex for DNA. Mifepristone and its analogues induce a dimerized receptor conformation with high affinity for DNA, while onapristone induces a conformation with low affinity for DNA. The clinical relevance of this mechanistic difference remains to be

elucidated. Structurally, mifepristone and onapristone differ in stereochemistry at C13, with onapristone being inverted from the usual steroid nucleus (Figure 5).

Estrogens are produced primarily in the ovaries and are responsible for stimulation of development of female sex organs, mammary glands, and various secondary sexual characteristics. Therapeutic uses of estrogen agonists include oral contraception (in combination with progestins), hormone replacement therapy in postmenopausal women, and treatment for dysmenorrhea, dysfunctional uterine bleeding, acne, hirsutism, failure of ovarian development, coronary artery disease, osteoporosis, and prostate cancers.

Steroidal estrogens (e.g., 17β-estradiol, estrone, Figure 6) have a characteristic phenolic A ring. Natural estrogens are deactivated in the liver; however, an α-substituent at C17 interrupts this metabolism (see

ethinyl estradiol). Several non-steroidal estrogens exist including flavinoids and di- and triphenylethylenes (e.g., diethylstilbestrol).

Currently identified anti-estrogens and partial estrogen agonists are predominantly triphenylethylenes. These include tamoxifen,⁷² chlomiphene, keoxifene, and 1 (Figure 6).⁷³ Steroidal anti-estrogens have been prepared and contain a long side chain at C₇ (e.g., ICI 164,384 and ICI 182,780).⁷⁴ Breast cancer is the current predominant therapeutic use for estrogen antagonists. Recent molecular insights into the transcriptional transactivating functions of estrogen receptor and other steroid receptors have opened new avenues for drug discovery, making possible the identification of compounds that demonstrate selectivity for a desired biological response.

The estrogen receptor has two distinct regions that confer transactivation of transcription: TAF-1 and TAF-2. Mutant forms of human ER (hER) have been constructed in which either TAF-1 or TAF-2 is genetically "excised". Cotransfection into mammalian cells of TAF-deleted hER genes or wild-type hER cDNAs, together with a plasmid containing an estrogen-responsive reporter gene such as luciferase cDNA, allows the rigorous analysis of the role played by TAF-1 and TAF-2 in the activation of transcription of various target genes by the ER. Using this cotransfection assay, it is possible to dissect the influences on ER-driven transcription of (i) cell background, (ii) promoter context, and (iii) activating ligand (various hER agonists, antagonists, and partial agonists).

When a particular ligand (e.g., estradiol, tamoxifen, or ICI 164,384) interacts with the ER, it induces (or stabilizes) a particular conformation of the receptor. Full agonists, such as 17 β -estradiol, induce a "fully active" conformation, in which both TAF-1 and TAF-2 are "exposed" and active. Full antagonists, such as ICI 164,384, appear to bind to ER (competitively with estradiol) and expose neither TAF-1 nor TAF-2. Partial agonists drive the ER into conformations "intermediate" between the fully active and fully inactive conformations driven by estradiol and ICI 164,384, respectively.

Interestingly, not all partial agonists drive the receptor into the same conformation. Tamoxifen appears to induce "exposure" of TAF-1 but not of TAF-2. Tamoxifen therefore functions as an agonist for TAF-1-dependent functions and as an antagonist for TAF-2-dependent functions. The former appears to underlie its estrogen-mimetic pharmacological effects in uterine tissue,⁷⁵ and the latter appears to account for its estrogen-blocking effects in breast cancer. Other compounds drive equally reproducible, partially-activating conformations of the ER, differing from that driven by tamoxifen. With the appropriate partial agonists for the ER, therefore, it is possible to achieve transcriptional enhancement of only a subset of estrogen-responsive genes. Compounds with a subset of estrogen's full spectrum of activities can be identified using assays based upon these principles.

Once exposed as a consequence of a ligand-induced conformational change in the ER, TAF-1 and TAF-2 can each independently enhance transcription of some estrogen-responsive genes, presumably by interacting with specific intracellular partner proteins. The recent identification of a 160 kDa estrogen receptor-associated protein (ERAP160)⁷⁶ lends further credence to this model. ERAP160 binds to ER through interactions

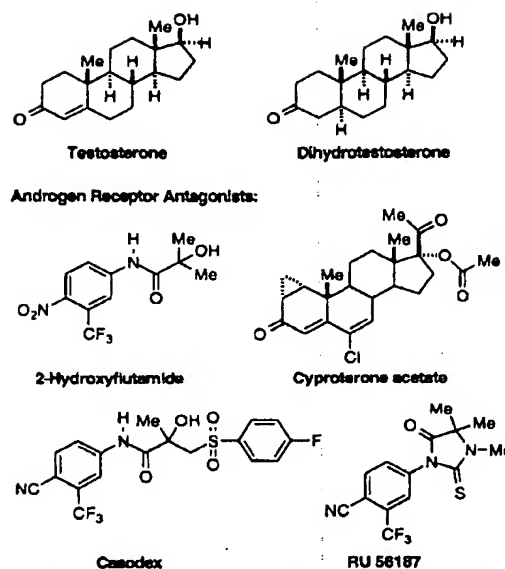


Figure 7. Androgen receptor agonists and antagonists.

involving TAF-2 in an estradiol-dependent manner. The binding of anti-estrogens to the receptor blocks the binding of ERAP160. It has been suggested that ERAP160 mediates transactivation of ER and that the ability of anti-estrogens to block ER-ERAP160 complex formation may account for their therapeutic effects in breast cancer. Although the identities of other postulated partner proteins remain unknown, indirect evidence indicates that their expression varies from cell type to cell type. The identification of these postulated partner proteins will provide yet another attractive target for the development of highly selective small-molecule drugs.

Emerging evidence supports the utility of an approach similar to that taken with ER and PR for the development of tissue-selective partial agonists for other receptors including PR, AR, MR, GR, and VDR.

Androgens are synthesized in the testes, adrenal cortex, and ovaries. The net effect of endogenous androgens reflects the combined actions of the secreted hormone, testosterone (Figure 7); its 5 α -reduced metabolite, dihydrotestosterone; and its estrogenic derivative, estradiol. Androgens serve different functions at different stages of male development and have clear therapeutic uses in the treatment of hypogonadism, growth retardation, breast carcinoma, and osteoporosis. The actions of androgens are mediated through AR.²¹⁻²³ Compounds that block the action or synthesis of androgens have proven useful in treatment of diseases such as prostate cancer, prostatic hypertrophy, hirsutism, male pattern baldness, and acne. Among the most potent orally active anti-androgens (Figure 7) is cyproterone acetate. This compound possesses progestational activity and suppresses the secretion of gonadotrophins, both of which are unwanted side effects. Other anti-androgens include flutamide, a prodrug for the active metabolite, 2-hydroxyflutamide,⁷⁷ casodex,⁷⁸ and an analogue of nilutamide.⁷⁹

Glucocorticoids and mineralocorticoids are steroid hormones produced by the adrenal cortex. Glucocorti-

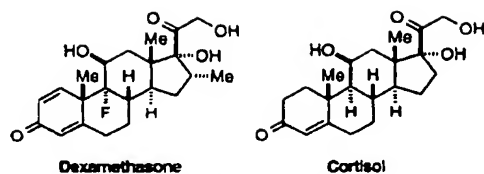


Figure 8. Glucocorticoid receptor agonists.

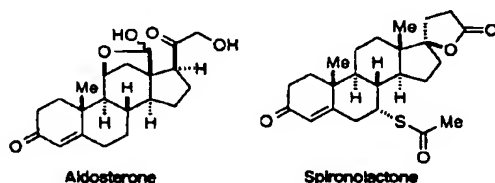


Figure 9. Mineralocorticoid receptor modulators.

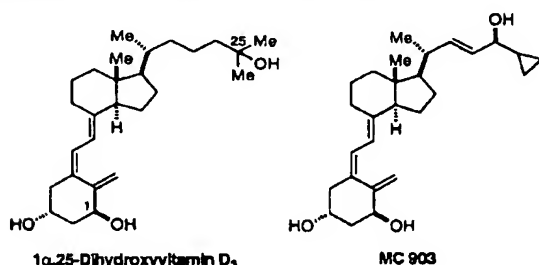


Figure 10. Vitamin D receptor agonists.

coids influence almost every organ and tissue in the body, affecting responses as diverse as behavior, immune function, and carbohydrate metabolism. Cortisol is the most potent naturally occurring glucocorticoid and stimulates or inhibits various biological functions. Synthetic glucocorticoid agonists can be divided into two groups: (i) 4-pregnene or 4-androstene derivatives and (ii) derivatives of cortisol or dexamethasone (Figure 8). It is not surprising, when the many actions of the glucocorticoids are considered, that these compounds actually have mixed agonist-antagonist activities when examined *in vivo*.

Mineralocorticoids (Figure 9) such as aldosterone regulate electrolyte balance in the kidneys, salivary glands, sweat glands, and gastrointestinal tract. Aldosterone acts by altering the ATP-dependent membrane transport of sodium and potassium ions. The action of aldosterone is inhibited by spironolactone and progesterone. Both act as competitive inhibitors of aldosterone by forming ligand-receptor complexes that are inactive.

A vitamin D₃ precursor is photochemically synthesized in the skin from 7-dehydrocholesterol and then undergoes a hydrogen shift to become vitamin D₃. As with hormones secreted by endocrine glands, this product is transported in the blood to distal sites where it is metabolized to the hormonally active form, calcitriol (1α,25-dihydroxyvitamin D₃, Figure 10), which then affects target tissues through interaction with the VDR, eventually resulting in increased plasma calcium concentrations. The conversion of vitamin D₃ to calcitriol occurring in the kidney is regulated by a negative-feedback control involving free calcium concentrations in the plasma.

The role of calcitriol in controlling the expression of a broad spectrum of genes is becoming increasingly evident. In addition to its active role in calcium homeostasis, calcitriol regulates genes associated with cell growth and tissue-specific structure. It is also responsible for maintaining the precise control of the concentration of calcium and phosphate ions in the plasma by modulating their absorption from the small intestine, enhancing their mobilization from bone and altering their excretion via the kidney. A growing body of evidence indicates that calcitriol also plays a role in the control of proliferation and differentiation of several cell types including epidermal keratinocytes. This effect of VDR agonists on skin has been utilized clinically in the treatment of psoriasis. Vitamin D analogues are also capable of causing differentiation of malignant cells, driving interest in VDR agonists in the treatment of leukemias and breast cancer.

All of the genomic effects of calcitriol are mediated by the VDR, which has been characterized biochemically from a number of tissues derived from many different animal species. There is evidence that certain rapid effects of vitamin D may reflect direct non-VDR-mediated actions at the plasma membrane. One compound that displays tissue-selective VDR agonist action is MC 903 (Figure 10),⁸⁰ which mimics the effects of vitamin D₃ on skin without increasing plasma calcium concentrations. It is not presently clear to what extent the tissue-selective actions of compounds such as MC 903 reflect intrinsic pharmacodynamics or pharmacokinetics or differential drug distribution. There is potential for other such tissue-specific vitamin D₃ partial mimics in the treatment of various skin diseases and cancers.

II. Intracellular Receptor Subtype Selective Compounds. Vitamin A (retinol) is derived exclusively from the diet as preformed retinol, retinyl esters, or carotenoids (provitamin A) and is stored primarily in liver as retinyl esters. Like vitamin D₃, retinol is transformed in the body to a variety of active metabolites that play important roles in several diverse cellular processes, including embryonic development, vision, reproduction, bone formation, hematopoiesis, metabolism, cellular differentiation, cellular proliferation, and programmed cell death.⁸¹

Retinal (vitamin A aldehyde) is required for retinal function. Other vitamin A derivatives, including *all-trans*-retinoic acid (ATRA or vitamin A acid), play an essential role in growth and differentiation of epithelial tissue and are necessary for reproduction, embryonic development, and bone growth. These actions of ATRA and related retinoic acid isomers (i.e., 9-*cis*-RA, discussed below) are mediated by RA IRs, which regulate gene expression.⁸²

The profound effects of retinol metabolites on cellular differentiation and proliferation have spurred the synthesis of thousands of RA analogues (retinoids) with potential use in a variety of skin disorders and malignant disease. Presently, the naturally occurring retinoids (Figure 11) ATRA (an active hormone) and 13-*cis*-RA (most likely acting by giving rise to ATRA and possibly 9-*cis*-RA) are used for the treatment of severe acne, while synthetic etretinate is prescribed for severe, refractory psoriasis. More recently, ATRA and 13-*cis*-RA have shown promise in the control of cancers or precancers such as acute promyelocytic leukemia,⁸³ head

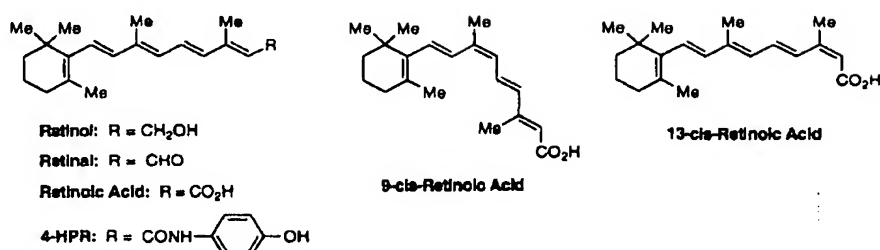


Figure 11. Retinoic acid and derivatives.

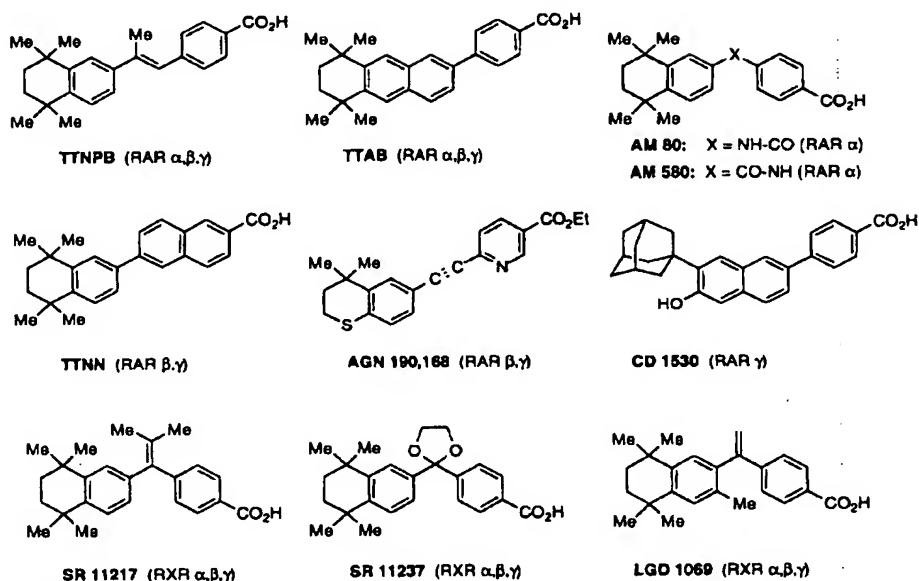


Figure 12. Synthetic retinoids.

and neck cancer,⁸⁴ and cervical dysplasia,⁸⁵ either as single agents or in combination with other agents such as interferon α . Other synthetic retinoids are in various stages of development for treatment of cancer and skin diseases, including *N*-(4-hydroxyphenyl)retinamide (4-HPR), which is in phase II trials as a chemopreventive agent in breast cancer treatment.⁸⁶ Retinamide is likely a prodrug for ATRA.

Unfortunately, widespread clinical use of the currently available retinoids is limited by undesirable side effects. These include mucocutaneous irritation, elevations in plasma triglycerides, headache, bone toxicity, and teratogenicity. The many diverse actions of retinoids, both desirable and undesirable, arise through activation of multiple retinoid receptor subtypes; thus retinoids with receptor subtype selectivity may have improved therapeutic indices.

To date six IR subtypes (or isoforms) that can be activated by ATRA in cells have been identified.⁸² Each receptor is encoded by a separate gene. Three of these, RAR α , RAR β , and RAR γ , are close genetic homologues. ATRA binds directly to each of the RARs, leading to activation of the RARs as transcription factors. The remaining three IRs responsive in cell culture to ATRA are members of the retinoid X receptor subfamily and are designated RXR α , RXR β , and RXR γ . The RXRs are close genetic homologues of each other, but are less

closely related to the RARs. Although the RXRs can be activated by ATRA in living cells, ATRA does not bind to the RXRs directly. ATRA activates RXRs indirectly upon conversion to 9-*cis* RA, the endogenous ligand for the RXRs, which binds to and activates both RXRs and RARs.⁸⁷ The pharmacological effects of 9-*cis* RA, the first novel non-peptidyl hormone described since vitamin D₃ was discovered in 1968, imply possible utility in the treatment of cancer and skin diseases. Chemically synthesized 9-*cis* RA (LGD1057) is currently in clinical trials in oral and topical formulations for cancer indications.

In addition to endogenous retinoids such as ATRA, 9-*cis*-retinoic acid and 13-*cis*-RA, synthetic, non-natural retinoids with novel retinoid receptor subtype selectivity are emerging as potentially exciting drugs. Investigators have used receptor binding and cotransfection assays to characterize known synthetic retinoids and newer analogues (Figure 12). Highly potent retinoids such as TTNPB and TTAB selectively activate the RAR subfamily but do not effectively distinguish among the isoforms.⁸⁸ Both AM-80 and AM-580 display selectivity for the RAR α isoform,⁸⁹ while TTNN is representative of compounds selective for the RAR β and RAR γ subtypes.^{88,89} Phase III clinical trials for topical treatment of acne and psoriasis have been completed using AGN 190,168, a novel RAR β - and RAR γ -selective compound.⁹⁰

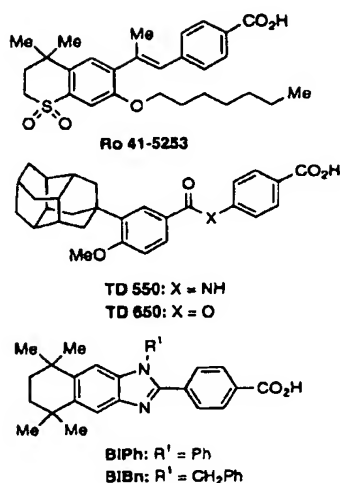


Figure 13. RAR antagonists.

CD 1530 is representative of structures reported to selectively activate the RAR γ subtype.⁹¹

Within the past 2 years, novel classes of RXR-selective retinoids have also been described. Both SR11217 and SR11237 display activation profiles for RXR α similar to that of 9-*cis*-RA at 100 nM.⁹² Neither compound activates RAR subtypes. Recently, a potent series of RXR-selective retinoids was developed by capitalizing on the observation that incorporation of a methyl substituent at the 3-position of the tetrahydronaphthalene moiety of TTNPB results in a retinoid with modest activity at all RAR and RXR subtypes.⁹³ A potent member of this new class, LGD1069, represents the first RXR-selective synthetic retinoid to enter clinical trials for the treatment of cancer.

Several RAR-selective antagonists have also recently been described. A series of sulfone derivatives related to Ro 41-5253 exhibits selectivity for the RAR α subtype (Figure 13).⁹⁴ Ro 41-5253 was shown to antagonize the teratogenic effects of the RAR α -selective agonist AM-580 in rat limb bud cell cultures and in mice. Additional RAR antagonists include TD550, TD650, BIPh, and BIBn, which inhibit retinoid-induced differentiation of human promyelocytic leukemia HL60 cells.^{95,96} As yet, no RXR-selective antagonists have been reported.

Data now emerging support separable biological roles for the various RAR and RXR subfamilies and individual subtypes in the control of cell proliferation, differentiation, and programmed cell death (apoptosis). RAR-selective compounds are sufficient to stimulate replication of human cytomegalovirus (hCMV) and induce the differentiation of an embryonal cell line that supports the growth of hCMV.⁹⁷ Further analysis of the pharmacological actions of the retinoids and identification of more selective analogues, in conjunction with studies using molecular and cellular biological approaches, are driving elucidation of the biological roles of the retinoid receptor subtypes and the delineation of the potential therapeutic uses of receptor subtype selective retinoids. Additional synthetic retinoids with useful patterns of selective interaction with the RAR and RXR subtypes hold great promise as pharmacological tools for biological investigations and as novel pharmaceuticals.

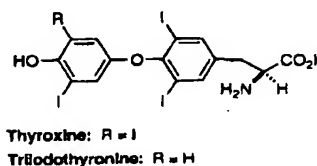


Figure 14. Thyroid hormones.

The thyroid gland is the source of two different thyroid hormones. Thyroxine (T₄) and triiodothyronine (T₃)⁹⁸ (Figure 14) are essential for normal growth and development and play an important role in controlling energy metabolism. Changes in the cardiovascular system are prominent consequences of the action of thyroid hormones. Stimulation of cholesterol metabolism to bile acids and lower plasma cholesterol levels result from elevated levels of thyroid hormones. A great many structural analogues of thyroxine have been synthesized in order to define structure-activity relationships, detect antagonists of thyroid hormones, or find compounds exhibiting desirable activity with reduced unwanted side effects.

Three TR subtypes, TR α 1, TR α 2, and TR β 1, have been identified in human tissues.⁹⁹ A fourth receptor subtype, TR β 2, was subsequently isolated from the rat¹⁰⁰ and has been identified in human tissues.¹⁰¹ The expression of the different TR subtypes is regulated both transcriptionally and post-transcriptionally. Each messenger RNA encoding a TR subtype shows characteristic patterns of developmental, tissue-specific, and hormonal regulation. The complexity of the TRs and their differing patterns of expression suggest that some of the myriad actions of thyroid hormone are mediated by specific TR subtypes. Such specific physiological roles of TR subtypes would imply better defined pharmacological effects for thyroid hormone agonists with TR subtype selectivity.

For other hormones of medical importance, including glucocorticoids and estrogens, receptor subtypes have not yet been identified, although in some senses MR can be thought of as a GR subtype at sites outside the kidney. Among the many orphan receptors (see below) and novel IRs that continue to be identified, currently unrecognized IR subtypes for known hormones may await identification.

III. Orphan Receptors. The ongoing discovery of orphan IRs continues to expand the list of superfamily members. The various orphan receptors are likely to play important functional roles, since (i) their sequences are highly conserved in mammals and even between phyla, (ii) they often have restricted spatial and temporal patterns of expression, (iii) transgenic animals in which various orphan receptors have been "knocked out" show functional impairment or lethality, and (iv) at least some of the orphan IRs can be implicated in the control of specific promoters (for example, hepatic nuclear factor-4 [HNF-4]). For the most part, the functional roles of these orphan IRs *in vivo* remain unknown. It is likely that some of these orphan receptors represent the signal-transducing receptors for currently uncharacterized endocrine, paracrine, or intracrine hormonal regulatory systems. Other orphan IRs may represent subtypes of receptors for known ligands. Thus the RXRs (originally considered orphans) bind and are activated by 9-*cis*-RA.⁸⁷

The restricted tissue distribution of some orphan IRs and the responses of these orphan receptors to known pharmacophores invite speculation about their possible functions. For example, orphan IRs, including chicken ovalbumin upstream promoter transcription factor (COUP-TF),¹⁰² COUP-TF β ,¹⁰³ and HNF-4,¹⁰⁴ and the three peroxisome proliferator activated receptor (PPAR) subtypes (PPAR α , PPAR β or NUC1, PPAR γ),¹⁰⁵ appear to be involved in the control of lipid, cholesterol, or lipoprotein metabolism, rendering them interesting from a pharmaceutical perspective. Known xenobiotics and drugs, such as the fibrate antihyperlipidemics, which induce peroxisome proliferation, appear to act through PPARs. Other orphan receptors, *e.g.*, nerve growth factor induced-B (NGFI-B)^{106,107} and the related Nur77 are expressed after cellular exposure to NGF or other growth factors and appear to influence cellular susceptibility to apoptosis. Although their function remains to be elucidated, the thyroid-related (TR2) orphan IR¹⁰¹ and its splice variants are expressed in a pattern essentially limited to the tissues of the genitourinary tract. Orphan IRs and other members of the superfamily also exhibit overlapping tissue and developmental distribution together with overlapping specificity for response elements within target genes. In these instances expression of target genes in a given tissue may be determined by interaction between members of the superfamily receptor complement present in that tissue.

Additional complexity is added with the identification of a growing number of IR subfamily members, as exemplified by the retinoid receptor family. The RXR subfamily was the first retinoid-related subfamily to be identified. Members of this subfamily form heterodimers not only with the retinoid-related RARs, but also with other hormone-activated and orphan IRs including VDR, TR, and PPARs. Recently, the RAR-related orphan receptors (RORs)¹⁰⁸ and retinoid Z-related receptors (RZR)¹⁰⁹ were identified. The RORs share common DNA- and putative ligand-binding domains, but differ in the N-terminal domains that are generated by alternative RNA splicing. Different members of this subfamily show different binding affinity for the RAR-related orphan response element (RORE) and, as a result, are able to mediate both constitutive and low-level transcription activation of target genes. RZR α and - β , although sharing a high degree of homology, have a different tissue distribution, with expression of RZR β confined to brain tissue. The RZR α s bind as monomers to natural and artificial retinoic acid response elements containing hexameric half-sites and are also able to form homodimers on selected response elements.

Lastly, there are orphan IRs that appear to be constitutively active in the absence of added ligands. These receptors may actually be responding to "intracrine" small-molecule ligands such as metabolic intermediates, *e.g.*, certain fatty acids to which PPARs respond. It is not necessarily the case that all orphan IRs have activating endogenous ligands. For example receptors such as COUP-TF and PR (in some species) can be activated by phosphorylation of appropriate residues by protein kinase A;¹¹⁰ the physiological and pharmacological relevance of such phosphorylation in modulation of IR activity remains to be definitively established. In any event the orphan IRs potentially

are novel targets for pharmaceutical intervention. The actualization of the potential inherent in the orphan IRs is a major challenge in IR-related drug discovery.

STATs and Drug Discovery. In addition to the steroid and small molecule hormones that control gene expression through interactions with IRs, there are peptide and protein ligands in the systemic circulation that produce alterations in gene expression in their target cells to which they bind. Included in this class of proteins are the cytokines (*e.g.*, the interferons and interleukins) and growth factors (*e.g.*, epidermal growth factor). The modulatory proteins are collectively termed extracellular signaling proteins (ESPs).⁴ ESPs cannot readily enter cells; they act by binding at the cell surface to specific receptors that span the cell membrane. Thus they can indirectly initiate a chain of events (characterized only recently for a growing number of ESPs) that culminate in changes in the pattern of cellular gene expression. The exact changes elicited are characteristic for the inciting ESP stimulus. For example, after a cell is exposed to interferon- α , specific genes are expressed yielding proteins that (i) render the cell more resistant to viral infection and (ii) reduce the rate at which the cell proliferates. These specific changes in gene expression following exposure of cells to ESPs are in many cases mediated by members of a newly discovered transcription factor superfamily called STATs.⁴

The STATs characterized to date range in molecular mass from roughly 80 to 113 kDa and are not genetic homologues of any other known group of proteins. Among the ESPs, which at least in part appear to exert their effects on cells through specific STATs,¹¹¹ are the interferons (IFNs) [IFN α , - β , and - γ]; the colony-stimulating factors (CSFs) [erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF), and probably the recently described thrombopoietin (Tpo)]; various interleukins (ILs) [IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13]; various growth factors [including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), oncostatin-M (OncoM), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF)]; and several peptidyl hormones (*e.g.*, growth hormone (GH) and prolactin).

The identification of the first STATs^{4,112} led to the elucidation of the biochemical events that mediate the changes in gene expression in response to interferons. Surprisingly, there is an underlying unity in the way that many additional ESPs, with their myriad of distinctive biological effects, act to control gene expression after binding to their cell surface receptors. In response to an ESP stimulus, specific STAT proteins are phosphorylated on tyrosyl residues within minutes after the binding of the ESP to its cell-surface receptor. This phosphorylation of STAT proteins is mediated by specific kinases called Janus kinases, or JAKs (see below) and results in their conversion from latent to active transcription factors.

To date, six different STAT family members (STATs1, -2, -3, -4, -5, -6) have been discovered, and as shown in Table 2 the cytokines that activate all of these STATs have been identified. However, it is highly likely that additional STATs will be found that participate in signal transduction of still other cytokines.

Table 2. Cytokines Utilizing the JAK/STAT Pathway

cytokine	STAT activation	JAK activation ^a
IFN α	STAT1, -2	JAK1, Tyk2
IFN γ	STAT1	JAK1, JAK2
IL-10	STAT1, -3	JAK1, Tyk2
IL-2	STAT5, -3	JAK1, -3
IL-7	STAT5, -3	JAK1, -3
IL-9	STAT5, -3	JAK1, -3
IL-15	STAT5, -3	JAK1, -3
IL-4	STAT6	JAK1, -3
IL-13	STAT6	JAK1
IL-3	STAT5	JAK2
IL-5	STAT1, -3	JAK2
GM-CSF	STAT5	JAK2
IL-6	STAT1, -3	JAK1, -2, Tyk2
IL-11	STAT3	JAK1, -2, Tyk2
LIF	STAT3	JAK1, -2, Tyk2
OSM	STAT1, -3	JAK1, -2, Tyk2
CNTF	STAT3	JAK1, -2, Tyk2
IL-12	STAT3, -4	Tyk2, JAK2
G-CSF	STAT3, -5	JAK1, -2
Epo	STAT5	JAK2
Tpo	STAT5	JAK2
prolactin	STAT5	JAK2
growth hormone	STAT1, -3, -5	JAK2
CSF-1/M-CSF	STAT1, -3	?
EGF	STAT1, -3, -5	JAK1
PDGF	STAT1, -3	?

^a A question mark (?) indicates that JAK activation has not been reported.

The colony-stimulating factors and interleukin-3 promote the growth of specific cell lineages within the bone marrow, giving rise to the mature cell types found in the blood. GM-CSF and G-CSF are currently used following cancer chemotherapy to increase the speed with which the white blood cell counts return to levels that are protective against infection or to facilitate the process of bone marrow transplantation in cancer patients. Epo has been extraordinarily successful in treatment of anemia due to renal failure. Interferon- β is used in the management of relapsing multiple sclerosis. Interferon- α is one of the cytokines produced by cells in response to viral infection. Recombinant IFN- α has been successfully utilized in the treatment of infectious hepatitis, hairy cell leukemia, and other cancers. The clinical utility of a variety of other cytokines is currently being assessed. The definition of the mechanisms by which the JAKs and STATs are activated and an understanding of their role in ESP signal transduction present new opportunities to discover orally bioavailable small molecule drugs mimicking or blocking medically important ESPs.

Mechanism of JAK/STAT-Mediated Signal Transduction

After an ESP binds to its cognate receptor, a cascade of events is initiated that leads to modulation of gene expression. The primary driver of this cascade appears to be protein phosphorylation. Receptor occupancy, probably through receptor dimerization, leads to changes in the cytoplasmic domain of the receptor that are "recognized" intracellularly. Evidence indicates that the altered receptor cytoplasmic domain becomes an effective "docking platform" for the appropriate members of a tyrosine protein kinase family known as the "Janus kinase" or JAK family¹¹³⁻¹¹⁵ and/or members of the

STAT family of latent transcription factor subunits. Assembly of an appropriate complex of JAKs and STATs anchored to the cytoplasmic domain of various ESP receptors results in tyrosyl phosphorylation and biochemical activation of particular JAKs. The activated JAKs then phosphorylate a subset of STAT proteins at a specific tyrosyl residue.

Although some receptors that utilize the JAK/STAT signaling pathway have intrinsic tyrosyl kinase activity (e.g., receptors for EGF and PDGF), most of these receptors do not. Furthermore all STAT phosphorylation is thought to be dependent upon the JAK kinases that associate noncovalently with the cytoplasmic domains of various ESP receptors. The receptors for ESPs are diverse and, with the exception of those for tumor necrosis factor (TNF) and IL-1, all seem to couple to the STAT signaling pathway through JAK activation. Receptors that bind ESPs as single chains (e.g., receptors for growth hormone, Epo, prolactin, and G-CSF) dimerize after ESP binding. This dimerization appears to lead to localization and activation of particular JAKs. Receptor multimerization also appears to be involved in JAK activation by ESPs that bind receptors with multiple chains. The receptors for one group of cytokines (IL-3, GM-CSF, and IL-5) are formed from different α chains and a common β chain. JAKs interact with the β chain. Lastly, there are cytokines (IL-6, LIF, OncoM, and CNTF) whose receptors are composed of specific α chains and a common protein component termed gp130. The action of these ESPs depends upon oligomerization of the gp130 subunit, which associates with specific JAKs, to activate the STAT signaling pathway.

Tyrosyl-phosphorylated STATs assemble into multimeric complexes, apparently stabilized by intermolecular interactions between src homology 2 (SH2) domains^{112,116} and phosphorylated tyrosyl residues within the STATs. SH2 domains, originally defined based on the src oncogene, are involved in binding to phosphotyrosine residues. A central region in the SH2 domain that includes the arginine residue that directly binds to phosphotyrosine is completely conserved among the STAT proteins. Different specific STAT complexes appear to be induced in response to different ESPs. These STAT-containing complexes move from the cytoplasm into the nucleus. Once in the nucleus, activated STAT complexes bind to specific response elements in the promoters of genes responsive to that ESP, acting as biochemically active transcription factors. Following ESP activation of JAK/STAT signaling, the cascade of events appears to be negatively regulated by the action of protein tyrosyl phosphate phosphatases. An important role for protein tyrosyl phosphate phosphatases in limiting ESP-mediated responses is implied by the ability of the protein phosphatases inhibitor vanadate to activate JAK/STAT-mediated transcription.¹¹⁷

JAK/STAT-mediated changes in the pattern of gene expression lead to alterations in levels of corresponding encoded proteins and therefore to altered cell function. As with hormone-induced IR-mediated changes in gene expression, the consequences of STAT signal transduction show a gradual onset (minutes to hours) and can last a relatively long time (hours to days). The STATs therefore exert their activity in a manner analogous to that of the IRs: the STATs transduce ESP signals that

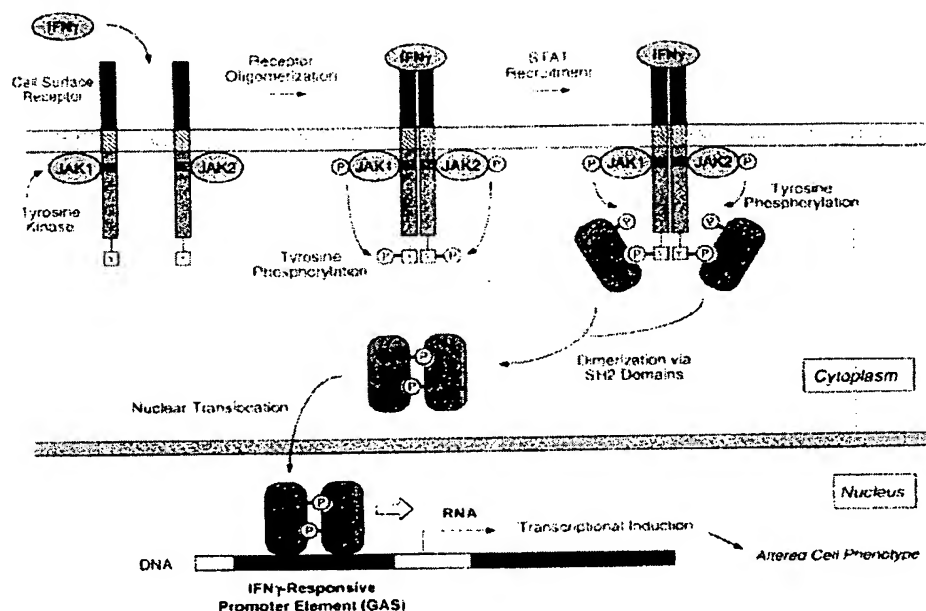


Figure 15. The signal transduction pathway utilized by interferon- γ (IFN- γ). Binding of IFN- γ to the IFN- γ receptor causes multimerization of the receptor and activation of JAK1 and JAK2. These activated JAKs phosphorylate STAT1. This leads to formation of the active transcription factor. After entering the nucleus, the STAT1 homodimer recognizes the IFN- γ activation sequence (GAS) in the promoters of IFN responsive genes and enhances transcription of those genes.

control gene expression, and the IRs serve a similar role for the non-peptidyl hormones.

The signal transduction pathway utilized by IFN- γ is shown in Figure 15. As described above, the first step in IFN- γ signal transduction is the binding of IFN- γ to its cell surface receptor. Receptor binding results in receptor oligomerization (a simplified depiction is shown in Figure 15), leading to activation of the receptor-associated JAKs, presumably by cross-phosphorylation. The receptor cytoplasmic domain is then in turn phosphorylated on tyrosine, presumably by the JAKs themselves. A single phosphorylated tyrosine residue on the receptor serves as a docking site for STAT1 (via its SH2 domain); STAT1 then becomes phosphorylated on a specific tyrosyl residue, again presumably via the JAKs.¹¹⁸ The phosphorylation of this STAT leads to its dimerization, yielding an active transcription factor. The STAT1 dimer can then move into the nucleus and bind to a DNA sequence element, known as an IFN- γ activation sequence (GAS), in the promoter of IFN- γ responsive genes. Binding of the STAT1 dimer to the promoter of these genes causes transcriptional activation in a manner analogous to the IRs. Although IFN- α -induced signal transduction is very similar to that of IFN- γ , the STATs activated are different as is the makeup of the DNA binding complex. Thus, IFN- α treatment leads to activation of JAK1 and tyk2 and phosphorylation of STAT1 and STAT2. Unlike IFN- γ and most other cytokines, the IFN- α -induced multimeric complex, termed interferon-stimulated gene factor-3 (ISGF3)^{4,119} also includes a DNA-binding protein, termed p48, that is not a STAT family member. In the absence of the activated STAT components, p48 shows only weak DNA binding. The ISGF3-STAT complex moves into the nucleus, specifically recognizes IFN- α stimulated response elements (ISREs) within the promoters of

genes responsive to IFN- α , and enhances the transcription of those genes.

The STATs and JAKs implicated in signal transduction of a variety of ESPs are shown in Table 2. Although there is significant overlap in the STATs participating in complexes induced by different ESPs, it is believed that selective action of a single ESP is obtained based upon the STATs and accessory proteins such as p48 forming the active complex, the overall transcription factor pool within the specific cell type, the precise nature of the STAT response element,¹²⁰ and the promoter context in which the STAT response element resides.

Discovery of Drugs Modulating ESP Action

Administration of specific ESPs (e.g., Epo, G-CSF, GM-CSF), various interferons, or IL-2 can have a medically beneficial effect. The therapeutically useful ESPs are often relatively difficult and expensive to manufacture, and they must be administered parenterally and frequently. In some pathological conditions, it may be highly desirable to specifically inhibit the actions of individual ESPs. Currently there are no small-molecule drugs known to act by directly modulating ESP-induced JAK/STAT-mediated signal transduction. However, the known physiological and pharmacological activities of many of the ESPs and their putative roles in the pathophysiology of various diseases suggest possible utilities for small-molecule ESP antagonists. Examples include possible use of interleukin or interferon antagonists in the treatment of inflammation or CSF inhibitors in treatment of leukemias.

A variety of assays can be considered in the construction of screens for compounds capable of modulating ESP-signaling pathways. Among these is a cellular

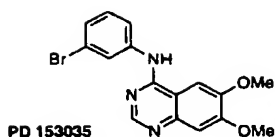


Figure 16. Inhibitor of epidermal growth factor receptor tyrosine kinase.

transcriptional assay similar to that used for intracellular drug discovery. In this approach a cellular background that harbors the required components of the signaling pathway could be used (*e.g.*, ESP receptor, JAKs, STATs, and tyrosine kinase phosphatases); alternatively, plasmid constructs capable of expressing one or more of the components of the signaling pathway (*e.g.*, ESP receptors, JAKs, or STAT components) can be introduced into the cell. A plasmid consisting of a promoter containing a STAT response element controlling transcription of a reporter gene such as luciferase is then introduced into the cell. Alteration in reporter gene transcription is then used to monitor the efficacy and potency of compounds tested in the screen.

Second, enzymatic assays for selected kinases and phosphatases can be established to identify compounds¹²¹ capable of directly affecting the activity of these critical components of the signaling pathway. In this regard, a very potent and selective inhibitor of the intrinsic tyrosine kinase activity of the EGF receptor has been identified¹²² (Figure 16), suggesting that sufficient structural heterogeneity may exist among tyrosine kinases to allow the discovery of selective and potent kinase inhibitors.

Finally, assays can be established that assess physical interactions of the components of the signaling pathway. These could include the interaction between the ESP receptor and the appropriate JAKs, the receptor and STATs, JAKs, and STATs, and homodimeric or heterodimeric STAT complexes.

Each of these approaches has potential advantages and disadvantages, but all could be used to identify novel small molecules capable of modulating the signal transduction pathway induced by selected ESPs. Drug discovery opportunities in this area are rapidly emerging. Small-molecule agonists could act in a variety of steps in the signaling pathway, including inhibition of phosphatase activity or stabilization of interactions between the various components of the pathway. The evidence cited above demonstrating that vanadate induces transcriptional activation of an ESP response element in the absence of the ESP suggests that JAK/STAT pathways are kept quiescent by the continuous action of protein tyrosyl phosphate phosphatases. Any compound that perturbs the activity or localization of these phosphatases would be expected to activate the pathway. In addition, nuclear protein tyrosyl phosphate phosphatases may be involved in the inactivation of phosphorylated STATs. Compounds that act by stabilizing protein-protein interactions, such as the immune suppressants cyclosporin and FK506 are precedents for the sorts of compounds that might stabilize STAT complexes.

Small-molecule ESP agonists could be therapeutically valuable in a variety of important disease states, for example by replacing (i) Epo for treatment of anemia; (ii) G-CSF, GM-CSF or Tpo as an adjunct to cytotoxic

cancer chemotherapy; (iii) IFN- α for induction of an antiviral state to treat infectious hepatitis; (iv) IL-2 or IFN- α for cancer therapy; and (v) growth hormone.

ESP antagonists could also act at several different points in the STAT-signaling pathway. These include binding to the cell-surface receptor, disrupting physical interactions that lead to receptor dimerization, preventing interactions between JAK and STAT molecules, interfering with interaction between JAK/STAT complexes and the general transcription complex, and specifically inhibiting JAK activity.

Antagonists of ESP signaling could be useful in inflammatory disease exacerbated by a variety of ESPs including the interferons IL-2, IL-4, and IL-6. IL-2 antagonists could be used in immunosuppressive therapy for graft rejection while an IL-4 antagonist could reduce allergic symptoms. EGF or PDGF antagonists have potential utility in treatment of growth factor dependent cancers. Thus, multiple drug discovery opportunities are represented by modulation of ESP action through the JAK/STAT signaling pathway.

Future Aspects

The progress made in understanding the mechanisms through which extracellular signals, such as hormones and cytokines, act to effect gene transcription has provided many new and interesting avenues for development of therapeutically important small-molecule drugs. As these signal transduction pathways are dissected further, the importance of receptor conformation, receptor interactions with accessory proteins, the roles of different subclasses of receptors, interactions between different IRs, and the role of orphan receptors in determining the specificity of the action of extracellular signals will become clear. Elucidation will potentially provide still more exciting routes for development of small-molecule drugs tailored to evoke a highly specific response.

Long-term therapy with known IR modulators is associated with detrimental side effects that limit their use. Definition of the consequences of ligand binding, in terms of alteration of receptor conformation, may be the key to overcoming this problem. Alterations in receptor conformation will affect both activation of specific transactivation domains within the receptor and interaction of these domains with other proteins required for transcription initiation. The development of screens that detect specific alterations in receptor conformation is critical to the identification of compounds with selective activity.

One approach is the use of assays that identify compounds capable of activating only a subset of receptor transactivation domains. Identification of cells and/or mutant receptors capable of distinguishing compounds that activate via specific transactivation domains can lead to the discovery of agonists or partial agonists with selective activity. The validity of this approach has been demonstrated for ER and may extend to other IRs. Alternatively, as we gain understanding of the role played by accessory factors in the regulation of transcription, assays designed to directly measure productive interaction between these factors and the IRs may aid in the identification of new classes of selective compounds. Recently, accessory proteins have been identified that associate with a number of different IRs.

These include the estrogen receptor associated proteins (ERAPs),⁷⁶ additional ER-associated proteins that are involved in the modulation of ER activity, the triiodothyronine receptor auxiliary protein (TRAP),¹²³ and the 110 kDa receptor accessory factor (RAF)¹²⁴ associated with AR. As these and additional, yet to be discovered, factors are characterized and their tissue distributions are determined, their physiological significance can be elucidated. Both functional and biochemical assays dependent on these proteins can then be established and used to discover compounds with selective therapeutic action.

The growing number of IRs with related subtypes provides another means to discover selective small-molecule modulators. To date, four distinct subfamilies of the retinoid receptor family have been identified: RAR, RXR, ROR, and RZR. Each of these subfamilies contains a variable number of subtypes: three RARs, three RXRs, two RZR, and two RORs. Subfamily-selective compounds, as well as pan agonists, for the RARs and RXRs have been identified. A variety of assays have demonstrated that retinoid subtype-selective compounds lead to different pharmacologies. By exploiting the existence of subtypes in other IR families, compounds may be found that also exhibit differences in pharmacology. The TR and PPAR receptor families both provide exciting targets with potential utility in the treatment of cardiovascular disease and obesity. Since fibrates, which are currently used to reduce triglycerides, modulate the activity of PPARs, more selective compounds may give cleaner pharmacology. PPAR γ is activated during the differentiation of adipocytes; modulating its activity may be important in controlling obesity. Thyroid hormone is clearly implicated in thermogenesis, a critical control point in fat deposition and use. In addition, thyroid hormone deficiency leads to elevated serum lipid levels that can be corrected by replacement therapy. The limited use of thyroid hormone in normal individuals due to its associated side effects may be overcome through the development of receptor-selective compounds.

The interaction between ligand-bound receptors and response elements in target genes may provide another means through which selectivity can be achieved. In general, target genes regulated by binding of the RARs and RXRs contain response elements that consist of two directly repeated half-sites. Recent studies have shown that RXR/RAR heterodimers activate transcription in response to *all-trans*-RA or 9-*cis*-RA by binding to direct repeats separated by five base pairs (termed a DR5 element) such that RAR occupies the downstream half-site. RXR homodimers activate transcription in response to 9-*cis*-RA by binding to direct repeats separated by one base pair (a DR1 element). RAR/RXR heterodimers can also bind to DR1 elements, with greater affinity than the RXR homodimer; however, in most contexts RAR/RXR heterodimers are unable to activate transcription in response to either *all-trans*-RA or 9-*cis*-RA. Thus, RARs appear to inhibit RXR-dependent transcription from these sites. RAR can be switched from a retinoid-dependent activator to an inhibitor when it occupies the upstream half-site of the DR1 element. These findings regarding the interaction between the ligand-bound receptors and their response elements clearly demonstrate that receptor conformation and

binding characteristics can be manipulated to alter the physiological outcome of receptor binding.

Not only is tissue distribution of the receptor itself a factor in restricted activity but also the availability of heterodimeric partners is critical in determining receptor activity. The role of heterodimer formation between subfamily members of the same IR family in the regulation of target genes has been demonstrated. A body of evidence now exists indicating that heterodimer formation between different intracellular families also plays an important role in the regulation of gene expression. The receptor complement of a particular cell or tissue type will therefore determine not only the response mediated by a given IR family, but may also significantly affect responses mediated by other intracellular superfamily members. As the role of heterodimer formation between superfamily members is examined, the tools developed will allow us to monitor these interactions, which in turn will enable identification of new drug targets.

Lastly, one of the most promising avenues for selective small-molecule drugs is the identification of modulators of the growing number of orphan receptors. The orphan receptors' tissue distributions and patterns of interactions with other IRs indicate that they play important roles in the regulation of gene transcription. Defining their physiological roles is the first step in exploiting these receptors for drug discovery. Identification of ligands, determination of their effects *in vivo*, and production of transgenic mice in which the gene for the orphan receptor is knocked out or overexpressed are ways that this can be accomplished.

The complexity of signal transduction pathways for IR ligands and for extracellular signaling proteins, such as the cytokines, can lead to the discovery of small-molecule modulators via numerous routes. The future challenge resides in the dissection of these pathways and in determining the optimal points of intercession for useful therapeutic outcome. For the IRs, this will require greater understanding of the proteins that transmit information from the IRs to the general transcription apparatus. A greater understanding of the role of IR phosphorylation may also be useful in the identification of new and useful targets for drug discovery. For the JAK/STAT pathways, further insight into the selective modulation of the activity of specific kinases and phosphatases, as well as the myriad protein-protein and protein-DNA interactions involved in signal transduction will be required. The ability to identify small molecules that modulate these activities in the IR and JAK/STAT pathways depends greatly upon the development of high-throughput screens based upon molecular insights into their mechanisms of action. Although this review describes drug discovery approaches based upon modulation of transcriptional activity, additional targets for modulation of steroid hormone action have been identified, particularly the steroid metabolizing enzymes that are known to both activate and inactivate receptor ligands.

Conclusion

From the perspective of drug discovery, the parallels between IR and STAT signal transduction are clear. Both the STATs and the IRs are latent transcription factors activated by cellular exposure to relevant ligands.

High-throughput, cell-based screens using reporter enzymes can be constructed in which the consequences of transcriptional modulation by potential small molecule agonists of STATs and IRs can be readily assessed. The structures of the reporter plasmids used in the IR and STAT assays are also similar. Each is composed of a reporter enzyme gene under the transcriptional control of a response element in the context of a minimal or naturally responsive promoter. These screens have demonstrated utility in IR drug discovery. The discovery of the JAK/STAT-signaling pathway presents an exciting approach to cytokine-related drug discovery that can yield small-molecule agonists and antagonists with patentability, oral bioavailability, and ease of manufacture. The drug discovery strategies described in this review are designed to identify compounds with novel and therapeutically useful properties.

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Quantitative assessment of spironolactone treatment in women with diffuse androgen-dependent alopecia

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Synopsis

From a group of 12 Caucasian females with diffuse androgen-dependent alopecia, six were treated for 12 months with spironolactone (75 or 100 mg per day) and six remained untreated. In the untreated (control) group, mean values for total hair density ($P < 0.05$) and meaningful hair density ($P < 0.01$) were significantly lower 12 months later. In contrast to these findings, no significant change in total hair density or meaningful hair density could be found in treated subjects. In two women the initial dose of spironolactone was doubled, and treatment continued for a further 12 months; in both cases increases in total hair density and meaningful hair density were observed. The androgenic hormonal variables all decreased on treatment. However, dihydrotestosterone and 3α -androstanediol-glucuronide levels were almost 50% higher. Low-dose spironolactone, 75 to 100 mg per day, appears capable of stabilizing the course of diffuse androgen-dependent alopecia in women. Initially, dosages > 150 mg per day may be necessary to improve hair quality and increase hair density. However, further long-term studies are required to confirm these findings.

INTRODUCTION

In women, thinning hair is predominantly a genetic condition (known as diffuse androgen-dependent alopecia, androgenic alopecia, androgenetic alopecia, common baldness, diffuse alopecia, diffuse hair loss, or female pattern baldness) that requires androgen-mediation for its phenotypic expression. The prevalence in women is frequently quoted to be around 30%, although precise epidemiological data are not available (1). The hair loss is typically diffuse, affecting the frontal and vertex areas with similar severity (2). Often a 1-2-cm band of denser hair is retained along the frontal hair line. A male-type pattern of hair loss, marked temporal or vertex recession, is less frequent (3). The major aesthetic change is the appearance of wider partings and a greater visibility of scalp through the hair. Changes in hair density may become apparent after

an episode of increased hair shedding, but for some an insidious and gradual change occurs over many years.

Diffuse alopecia in women has been associated with endocrine abnormalities, but a relationship cannot be established in all cases. Thirty per cent or more have no demonstrable endocrine abnormality (2,4-7). In Europe, the anti-androgen cyproterone acetate (CPA) has been available for many years and, in combination with ethinyl estradiol (EE₂), has been successfully employed to treat acne, androgen-dependent alopecia, and hirsutism. Cyproterone acetate has never been available within the USA, and therefore an alternative anti-androgen was sought. In the late sixties, the aldosterone antagonist spironolactone was reported to have anti-androgen activity (8-10). Subsequent studies demonstrated its usefulness in the treatment of hirsutism (11-15) and acne (16, 17), but only anecdotal evidence exists for androgen-dependent alopecia (17, 18).

Three fundamental hair variables—hair density, hair diameter, and hair length—can quantitatively characterize most scalp hair disorders. The unit area trichogram (19) is a technique capable of providing such information (20). This method has been employed to detail scalp hair changes during systemic anti-androgen therapy in women (21, 22) and topical 2% minoxidil treatment in men with male pattern baldness (23). In view of the anti-androgen activity associated with spironolactone, we used the unit area trichogram to evaluate scalp hair in women with diffuse androgen-dependent alopecia treated for up to 24 months.

METHODS AND MATERIALS

SELECTION OF SUBJECTS WITH DIFFUSE ALOPECIA

Twelve premenopausal Caucasian females, mean age 37 years (range 30-45 years), with diffuse androgen-dependent alopecia, participated in this study. Each had noticed cosmetically thinner hair for at least 36 months prior to entering the study. All gave their informed consent. No subject had suffered any illness lasting longer than seven days, nor had they taken prescribed medications (including oral contraceptives) or applied products known to influence hair growth for six months prior to entering the study. None had been pregnant within the previous two years, and subsequent thyroid evaluations were all normal. None had been referred to an endocrine unit for obvious androgen excess or sought medical help for acne or hirsutism. Subjects with alopecia areata, cicatricial alopecia, or a history of thyroid dysfunction were excluded, as were those who exhibited alopecia of the male-type pattern.

ALLOCATION OF CONTROLS AND SUBJECTS TREATED WITH SPIRONOLACTONE

The treated group was comprised of six subjects, mean age 35 years (range 30-41 years), with total hair densities between 162 and 336 hairs per cm², who elected to undergo spironolactone therapy (75 or 100 mg per day) for 12 months. The control group was comprised of six subjects, mean age 38 years (range 30-45 years), matched for total hair density, who elected to remain untreated for 12 months. The duration of alopecia ranged from 3 to 16 years and was similar between and within the two groups. There was no significant difference between the mean age of control or treated subjects (unpaired

Student's t-test). After 12 months, two subjects from the treated group agreed to continue spironolactone therapy, employing higher dosages (150 or 200 mg per day), for a further 12 months.

THE UNIT AREA TRICHOGRAM AND REPRODUCIBILITY OF HAIR VARIABLES EMPLOYED

All subjects followed the same standardized shampooing procedure one month prior to sampling, which required the hair to be shampooed daily but not on the morning of sampling. Hair samples were obtained from the frontal area basally and, within 5 mm of the original sites, 12 or 24 months later. The mean area sampled was 56 mm² basally and 55 mm² after 12 months. From two unit area trichograms, separated by less than 25 mm (Figure 1), values for hair variables were obtained from each site. The generated data were pooled, and the difference (as a %) between this pooled value and an individual site provided a measure of the reproducibility. The sum of these differences gave a mean value for the group, which was <5% for each variable. The term *meaningful hair* was defined as all non-vellus hair. A vellus hair had been characterized previously as a hair ≤ 30 mm in length having a diameter ≤ 40 μ m (23). The proportion of hair ≤ 80 mm in length provided an estimate of the disturbance occurring to the hair cycle during the previous six months. The percentage of telogen hair ≤ 30 mm in length was employed to assess complete hair growth cycles of less than six months. Where reference is made to hair length, this term relates to uncut hair only or cut hair above the pre-assigned limit. Dysplastic or dystrophic hair was classified as detailed previously (2), although their occurrence was less than 4%. Hairs in the catagen growth phase were grouped with the telogen population for data analysis. The mean number of hairs examined per unit

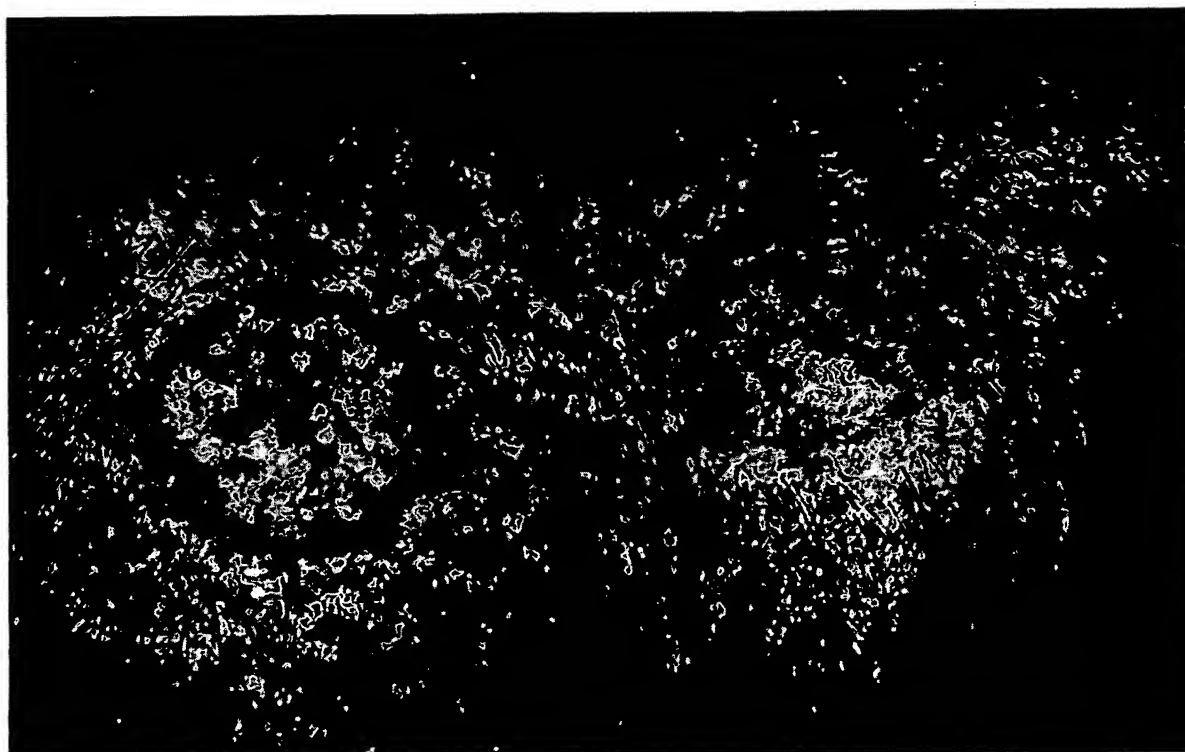


Figure 1. Two unit area trichograms separated by less than 25 mm.

area trichogram was 135 from controls and 141 from treated subjects. A total of 6624 hairs was examined in this study.

BIOCHEMICAL AND MEDICAL INVESTIGATIONS

Full medical, biochemical, haematological, and hormonal evaluations were undertaken in all subjects basally, on day 21 of the menstrual cycle and before 11.00 h following a 12-hour fast. Biochemical and haematological investigations were repeated after 6, 12, or 24 months as appropriate. All hormonal evaluations were undertaken by the Nichols Institute, San Juan Capistrano, California. The biochemical and hematological investigations were performed by MetPath, Teterboro, New Jersey.

STATISTICAL ANALYSIS

Group mean differences were compared statistically by Student's t-test for paired or unpaired samples as appropriate. All analyses were undertaken on an Apple Macintosh™ computer using the statistical program STATWORKS.™

RESULTS

Initially, in the treated group, irregular periods and mild hirsutism were noted in two subjects, while all six subjects had mild facial acne (two of whom suffered premenstrually). In the control group, two subjects had irregular periods, one had mild hirsutism, and two had mild facial acne (one premenstrually). None of these conditions were troublesome enough to warrant any subject seeking medical advice for these complaints. On treatment, two subjects with irregular cycles became regular, while two with regular menses developed irregular cycles. However, no subject withdrew from therapy because of any side effects.

COMPARISON BETWEEN BASAL HAIR VARIABLES AND THOSE OBTAINED AFTER 12 MONTHS IN CONTROL AND TREATED SUBJECTS

Mean baseline values for hair variables from controls and treated subjects were compared to values obtained from the same sites 12 months later (Tables I and II). In the control group, but not in the treated group, a significant decrease in total hair density ($P < 0.05$) and meaningful hair density ($P < 0.01$) was observed after 12 months (Figure 2). Subjective impressions supported these findings, particularly within the control group, where deterioration in hair quality was cause for concern.

BASAL BIOCHEMICAL AND HORMONAL EVALUATIONS FROM CONTROL AND TREATED SUBJECTS WITH DIFFUSE ANDROGEN-DEPENDENT ALOPECIA

Basal hematological and non-hormonal biochemical values were all within the reference range of the laboratory. The principle androgenic hormones, androstenedione, dehydroepiandrosterone-sulphate (DHEA-S), and total testosterone were also within their

Table I
Comparisons Between Hair Variables From the Frontal Area of Untreated Controls Basally and After 12 Months: Mean \pm SD and Range (in parentheses)

Variable	Control group (n = 6)	
	Basal	12 Months
Total hair density (Hair per cm ²)	231 \pm 57 (157-317)	* 206 \pm 72 (120-303)
Meaningful hair density (Non-vellus hair per cm ²)	191 \pm 67 (116-278)	** 169 \pm 74 (80-268)
(%) Anagen hair	79.4 \pm 9.3 (61.0-84.7)	ns 75.3 \pm 12.4 (56.9-93.8)
(%) Meaningful anagen hair	85.9 \pm 4.5 (81.5-94.2)	ns 79.0 \pm 10.7 (65.5-93.6)
(%) Hair \leq 80 mm in length	68.4 \pm 24.0 (25.9-89.0)	ns 74.5 \pm 14.9 (50.0-89.1)
(%) Vellus hair	17.9 \pm 16.4 (2.0-46.3)	ns 19.2 \pm 16.8 (2.0-50.8)
(%) Telogen hair \leq 30 mm in length	11.7 \pm 11.1 (2.0-32.9)	ns 11.2 \pm 9.1 (0.0-27.7)

ns = Not significant: $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ (paired Student's t-test).

respective reference ranges. The peripheral markers for hyper-androgenism were raised in six (50.0%) for free testosterone, in two (16.7%) for 3α -androstenediol-glucuronide (3α -A-diol-G), and in one (8.3%) for dihydrotestosterone (DHT). However, no correlation between individual or grouped hormonal values with any hair value could be established.

TRICHOLOGICAL AND HORMONAL CHANGES IN TWO SUBJECTS WHO CONTINUED WITH SPIRONOLACTONE THERAPY FOR A FURTHER 12 MONTHS

One subject who initially received 75 mg of spironolactone per day was increased to 150 mg per day, and one initially receiving 100 mg per day was increased to 200 mg per day. After 12 months of treatment with these higher dosages, both had increases in total hair density and meaningful hair density and a corresponding decrease in the percentage of vellus hair and telogen hair \leq 30 mm in length (Table III). Comparisons between basal hormonal levels and those observed after 24 months showed a substantial reduction in the androgenic hormones. However, both DHT and 3α -A-diol-G levels were inexplicably elevated.

DISCUSSION

The major initiating feature of androgen-dependent alopecia in men and women is the action of androgens upon the pilo-sebaceous unit (2,7,24,25), the associated dermal/sub-dermal tissue (26), and the receptor-binding phenomenon within the hair cell (27).

Table II
Comparisons Between Hair Variables From the Frontal Area Basally and After 12 Months of
Spironolactone (75 or 100 mg per day) Treatment: Mean \pm SD and Range (in parentheses)

Variable	Treated group (n = 6)		
	Basal		12 Months
Total hair density (Hair per cm ²)	256 \pm 78 (162-336)	ns	239 \pm 72 (145-313)
Meaningful hair density (Non-vellus hair per cm ²)	197 \pm 67 (123-276)	ns	189 \pm 51 (131-256)
(%) Anagen hair	78.5 \pm 5.1 (72.9-84.8)	ns	79.8 \pm 4.3 (74.8-84.7)
(%) Meaningful anagen hair	81.2 \pm 6.0 (70.2-86.2)	ns	79.5 \pm 9.9 (63.7-91.2)
(%) Hair \leq 80 mm in length	56.9 \pm 16.6 (37.6-80.8)	ns	56.1 \pm 18.8 (33.0-82.9)
(%) Vellus hair	23.2 \pm 6.0 (16.5-30.3)	ns	19.6 \pm 8.5 (9.6-33.3)
(%) Telogen hair \leq 30 mm in length	10.3 \pm 2.9 (6.8-13.9)	ns	10.9 \pm 5.6 (6.4-21.4)

ns = Not significant: $P > 0.05$ (paired Student's t-test).

Compounds with anti-androgen activity would therefore appear essential in the treatment of androgen-dependent alopecia (25), and studies employing CPA in combination with EE₂ support this view (21,23). For spironolactone, however, no quantitative data for alopecia were available; consequently, we employed dosing regimens established in acne and hirsutism. In moderately hirsute women, 50 mg of spironolactone per day was reported to regress terminal hair to vellus hair within six months of starting treatment (14). Good results had also been reported for dosages between 150 mg and 200 mg per day in acne (16) and hirsutism (13,17,28). However, with dosages >100 mg per day, side effects began to appear, and several studies reported drop-out rates in excess of 25% (12,15).

In our study, subjects treated with spironolactone (75 or 100 mg per day) had stabilized total and meaningful hair densities 12 months later (Table II), while in untreated controls a significant decrease was observed at this time (Table I). Subjective impressions supported these findings, particularly within the control group, where deterioration in hair quality was cause for concern. When the dosage was doubled, increases in total and meaningful hair densities were recorded in the two subjects so treated (Table III). Both were aware of an improvement in hair quality. In comparison to these findings for spironolactone, low-dose CPA-EE₂ studies suggest that CPA dosages ≤ 50 mg per month are unable to prevent further expression of the alopecia, whereas CPA dosages ≥ 500 mg per month are able to do so. Whether the degree of improvement achieved with high-dose spironolactone is similar to that obtained with high-dose CPA is the subject of an ongoing study.

One of the major problems in treating androgen-dependent alopecia is the difficulty in

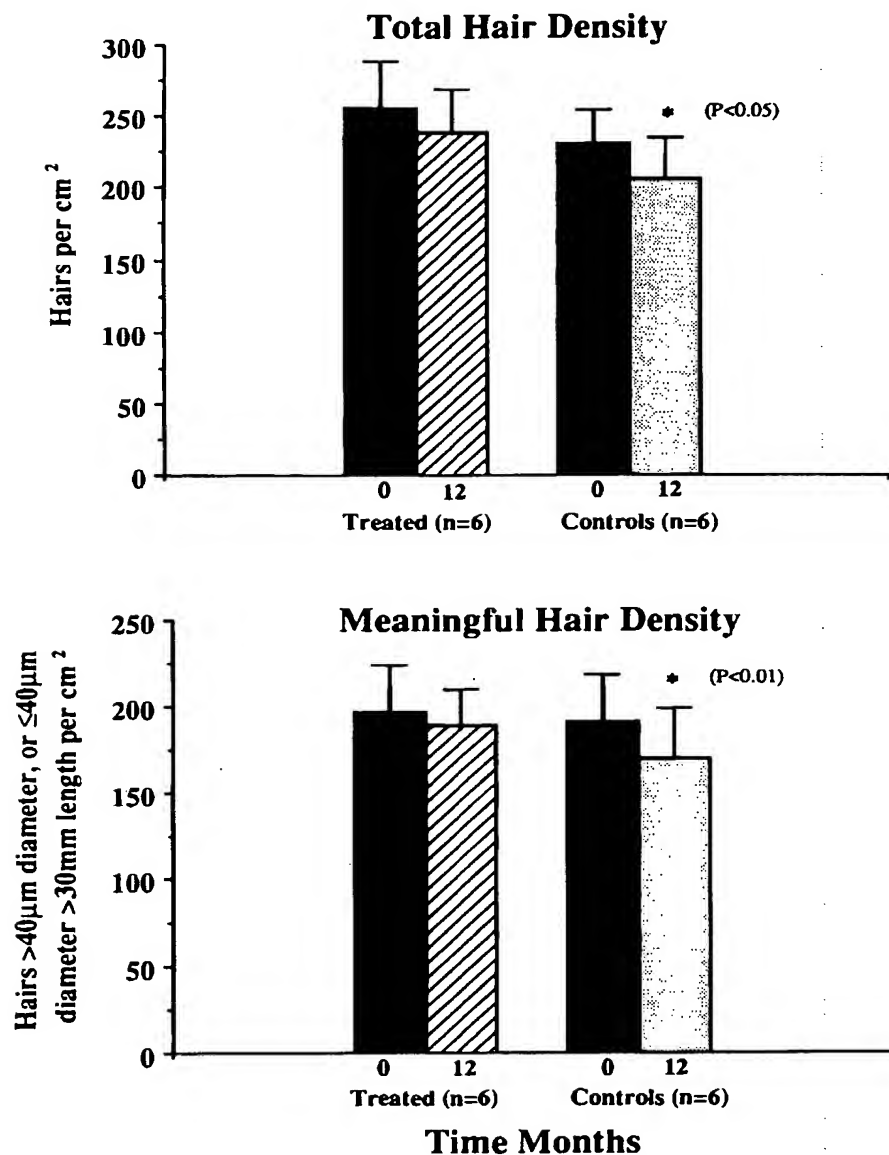


Figure 2. Changes in total and meaningful hair densities (mean \pm SEM) in controls and subjects treated with spironolactone (75 or 100 mg per day). *Significance level (paired Student's t-test).

predicting what effective regimen is required for a specific group or individual. Circulating hormonal levels do not provide any reliable information about the rate of change occurring in scalp hair or the dosage required to treat it (2). This is of considerable importance since the treatment time required may be several years. Moderate dosages of spironolactone (75 or 100 mg per day) appear capable of stabilizing the course of androgen-dependent alopecia, at least in these women, without any significant side effects. Initially, higher dosages may be necessary to improve hair quality and achieve increases in hair density. However, further long-term studies are required to confirm these findings. Our data for spironolactone supports the role of androgens as a mediating factor in genetic hair loss in women.

Table III
Comparisons Between Basal Hair Variables and Those Obtained After 12 and 24 Months of Spironolactone Treatment

Spironolactone (mg per day) Time (months)	Subject One			Subject Two		
	0	12	24	0	12	24
Hair variable						
Total hair density per cm ²	310	303	349	162	145	173
Meaningful hair density per cm ²	258	267	293	130	131	147
(%) Anagen hair	72.9	76.6	86.6	79.4	84.0	77.6
(%) Meaningful anagen hair	79.5	78.6	86.5	85.6	84.7	80.2
(%) Vellus hair	16.5	16.5	11.9	19.6	9.6	15.0
(%) Telogen hair ≤ 30 mm	13.2	6.2	2.6	11.2	6.4	7.5

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Topics in Primary Care Medicine

Approach to Patients With Hirsutism

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Hirsutism is a common medical condition that in most women is due to the polycystic ovary syndrome or is idiopathic. For a few women, hirsutism signals a serious underlying disorder such as an ovarian or adrenal tumor, congenital adrenal hyperplasia, or Cushing's syndrome. A detailed medical history and examination can identify women in whom a serious disease is suspected and for whom laboratory evaluation is warranted. Measurements of serum testosterone, dehydroepiandrosterone, and 17 α -hydroxyprogesterone levels, and 24-hour urinary cortisol concentrations are important screening tests. Therapy is directed at suppressing ovarian or adrenal androgen production, inhibiting the conversion of testosterone to dihydrotestosterone, or antagonizing the effects of androgens at the receptor level. (Sakiyama R: Approach to patients with hirsutism. *West J Med* 1996; 165:386-391)

Hirsutism is a common clinical condition affecting about 5% of women in the United States.¹ All too often, physicians dismiss hirsutism as a concern that does not deserve their clinical acumen. Perhaps this response is due to a lack of knowledge of the proper evaluation and management of hirsutism or the notion that hirsutism is merely a cosmetic problem. For most women, hirsutism is not a manifestation of a serious disorder, but rather a possible source of emotional disability. For some women, hirsutism is a warning sign of a more serious underlying problem. Appropriate management can be determined after a carefully directed history, physical examination, and limited laboratory testing.*

Hirsutism is defined as the excessive growth of androgen-responsive terminal hair in women. Therefore, for most women, hirsutism is a cutaneous manifestation of androgen excess. True hirsutism must be distinguished from hypertrichosis—that is, excessive growth of vellus or non-androgen-responsive hair. Vellus is fine, downy hair that is usually unpigmented. Terminal hairs are dark, thick, and found in the sex hormone-responsive areas of the pubis, axilla, back, face, chest, and abdomen. Androgens promote an increase in the number and thickness of terminal hairs in these areas. Women with androgen-dependent hirsutism either have exposure to excessive androgens or manifest a heightened androgen-receptor sensitivity to normal circulating levels of androgen. For other women, hirsutism is a non-androgen-dependent abnormality—that is, an adverse effect to certain drugs or a genetic characteristic, such as in women of Mediterranean or East Indian origin.

Testosterone and dihydrotestosterone are true andro-

gens as defined by their ability to interact with the androgen receptor. Testosterone binds to cell receptors, enters the cell cytoplasm, and is converted to dihydrotestosterone by a 5 α -reductase enzyme. Dihydrotestosterone, in turn, is bound to specific cytoplasmic receptors. Potential androgens are dependent on a conversion to testosterone and include dehydroepiandrosterone (DHEA) and androstenedione. Dehydroepiandrosterone is the major adrenal androgen that can be converted to testosterone, or sulfated to DHEAS before secretion. Measuring the DHEAS level is the preferred plasma test of adrenal androgen production because DHEA shows a circadian and menstrual rhythm not found with DHEAS. Androstenedione is the major C19 steroid produced by the ovaries, although the ovaries are also able to secrete testosterone and dihydrotestosterone. In normal women, two thirds of plasma testosterone is derived from the adrenal cortex through peripheral formation from DHEA and androstenedione.

Causes of Hirsutism

Causes of hirsutism can be classified as ovarian, adrenal, drug-related, idiopathic, or genetic (Table 1).² Ovarian disorders include the polycystic ovary syndrome and ovarian tumors. Adrenal disorders include congenital adrenal hyperplasia, Cushing's syndrome, and adrenal tumors. Hirsutism may also occur with the ingestion of anabolic steroids or as an adverse effect of a limited group of medications (see Table 1). Idiopathic hirsutism is defined by normal androgen concentrations and a lack of any identifiable underlying disorder. It has been proposed that idiopathic hirsutism results from an increased androgen-receptor sensitivity to normal androgen levels. More than 95% of women with hirsutism are found to have either idiopathic hirsutism or the polycys-

*See also the editorial by S. J. Agarwal, MB,BS, and H. L. Judd, MD, "What We See Most, We Understand Least," on pages 392-393 of this issue.

ABBREVIATIONS USED IN TEXT

ACTH = adrenocorticotrophic hormone, corticotropin
 CT = computed tomography
 DHEA = dehydroepiandrosterone
 DHEAS = sulfated form of DHEA
 FDA = Food and Drug Administration
 FSH = follicle-stimulating hormone
 GnRH = gonadotropin-releasing hormone
 LH = luteinizing hormone
 MRI = magnetic resonance imaging
 SHBG = sex hormone-binding globulin

tic ovary syndrome.³

The polycystic ovary syndrome is the most common identifiable cause of androgen hypersecretion in women. The underlying defect in this syndrome is postulated to be a nontumorous dysfunction of luteinizing hormone (LH) hypersecretion, with a subsequent stimulation of thecal and stromal ovarian cells to produce androgens. The polycystic ovary syndrome encompasses a wide spectrum of clinical manifestations. Patients may present with classic "polycystic ovary disease"—obesity, hirsutism, anovulation, and enlarged multicystic ovaries. On the opposite end of the spectrum, women with mild polycystic ovarian syndrome may not be obese and have regular ovulatory cycles and normal ovaries, with only subtle hormonal aberrations and hirsutism. Luteinizing hormone levels are frequently elevated in the polycystic ovary syndrome, but often to such a modest degree that its absolute value remains within the normal reference range. If the ratio of LH to follicle-stimulating hormone (FSH) is measured, however, it is frequently increased to 2.5 or greater in women with the polycystic ovary syndrome (most normal ovulatory women have an LH:FSH ratio of 1.0). Total plasma testosterone levels are elevated in 40% to 60% of women with this syndrome. In the other women, levels of total testosterone are normal, but those of free testosterone are elevated. Free testosterone represents the nonprotein-bound hormone that is thought to most closely reflect cellular hormonal actions. The major binding protein for testosterone is sex hormone-binding globulin (SHBG), which is decreased in women with the polycystic ovary syndrome and accounts for elevated levels of free testosterone despite normal total testosterone levels.

Ovarian and adrenal tumors are uncommon causes of hirsutism. Adrenal carcinomas are typically large by the time they produce excessive androgens. Adrenal adenomas can produce androgens and are typically small and difficult to localize. Ovarian tumors include Sertoli-Leydig cell tumors, hilar cell tumors, lipoid cell tumors, and adrenal rest tumors. Elevations in DHEAS levels suggest an adrenal origin of androgen production, whereas greatly elevated testosterone concentrations signal a possible ovarian or adrenal tumors.³

Cushing's syndrome is caused by the chronic overproduction of glucocorticoids and, to a lesser extent, adrenal androgens. Skin findings due to excessive androgen production include hirsutism, acne, and temporal hair recession, in addition to glucocorticoid-associated

TABLE 1.—Causes of Hirsutism*

General	Specific Cause
Ovarian	Polycystic ovary syndrome Neoplasms Sertoli-Leydig cell tumors Hilar cell tumors Lipoid cell tumors Adrenal rest tumors
Adrenal	Congenital adrenal hyperplasia 21-Hydroxylase deficiency 11 β -Hydroxylase deficiency 3 β -Hydroxysteroid dehydrogenase deficiency Cushing's syndrome Neoplasms Adrenal carcinoma Adrenal adenoma
Drugs (proprietary name)	Cyclosporine (Sandimmune) Danazol (Danocrine) Phenytoin (Dilantin) Glucocorticoids Minoxidil (Loniten) Diazoxide (Hyperstat) Anabolic steroids
Idiopathic	
Genetic	

*From Carr.³

findings of thin skin and purple striae. Classic congenital adrenal hyperplasia presents in infancy and is due to a deficiency of 21-hydroxylase, 11 β -hydroxylase, or 3 β -hydroxysteroid dehydrogenase adrenal enzymes. An attenuated or nonclassic form of congenital adrenal hyperplasia may present in adolescence or young adulthood.⁴⁵ A partial deficiency of the 21-hydroxylase enzyme is the most common form of nonclassic congenital adrenal hyperplasia. Decreased 21-hydroxylase activity leads to increased concentrations of 17 α -hydroxyprogesterone, which in turn is converted to testosterone.

Idiopathic hirsutism is found in 50% of women evaluated for hirsutism. Idiopathic hirsutism is defined by normal physical and laboratory findings in women with the onset of excessive hair growth in puberty or young adulthood. They do not have signs of virilization and manifest a slow progression of their hirsutism. Increased sensitivity of the hair follicles to normal circulating androgens is thought to cause the hirsute state.

History and Physical Examination

The primary goal of the history and physical examination is to identify patients in whom hirsutism is the cutaneous manifestation of a serious underlying disease. Hirsutism due to the polycystic ovary syndrome or idiopathic causes typically begin between the ages of 15 and 25 years and progresses slowly. Symptoms starting later in life, coupled with a rapid progression, suggest a more serious underlying disorder, such as an ovarian or adrenal tumor or Cushing's syndrome. The exception is peri-

menopausal women who may have more hair growth in their perimenopausal years due to an increased production of adrenal androgens. Women with greater elevations in androgen levels have virilizing symptoms such as balding, deepening of the voice, increased libido, or decreased body fat. Virilization is not typical of idiopathic hirsutism or the polycystic ovary syndrome and suggests a more serious disorder. Women should be carefully questioned about symptoms associated with specific underlying diseases. For example, those with the polycystic ovary syndrome may have amenorrhea, oligomenorrhea, infertility, or obesity. A recent weight gain with a cushingoid body habitus and hypertension suggests Cushing's syndrome, whereas flank pain or a mass coupled with weight loss may be due to an adrenal cancer. Finally, the use of prescribed medications or anabolic steroids should be carefully reviewed.

The examination is directed at documenting the extent and distribution of terminal hair and uncovering any clues as to an underlying cause. Hirsutism can be quantitated by the use of the Ferriman and Gallwey score.⁶ The extent of hirsutism is evaluated in nine body areas—upper lip, chin, chest, upper arms, upper abdomen, lower abdomen, thighs, and upper and lower back—and each area is given a point score of 0 (no hirsutism) to 4 (overtly virile). A total score of 8 or higher is consistent with the diagnosis of hirsutism. Physical findings of virilization include receded temporal hair, increased laryngeal size, decreased body fat, atrophied breasts, masculine musculature, and clitoromegaly (a clitoris of greater than 1.0 cm in diameter). Patients with Cushing's syndrome may have acne, purple striae, or centripetal obesity. A bimanual pelvic examination is done to exclude an ovarian mass, which is found in about half of women with ovarian tumors, or enlarged ovaries (polycystic ovary syndrome).

Laboratory Evaluation

In women who are presumed to have the polycystic ovary syndrome or idiopathic hirsutism, only a limited laboratory evaluation is required. For women with suspicious historical or physical findings, a more extensive laboratory evaluation is done to exclude androgen-secreting adrenal or ovarian tumors, Cushing's syndrome, or the nonclassic form of congenital adrenal hyperplasia. The two most important screening tests are measurements of total serum testosterone and DHEAS levels. Testosterone levels of greater than 7 nmol per liter (2.0 ng per ml) suggest the presence of an androgen-secreting ovarian or adrenal tumor, and DHEAS levels of greater than 19 μ mol per liter (700 μ g per dl) suggest an adrenal tumor.⁷ Elevations of testosterone and DHEAS levels to this degree are not specific for a neoplastic cause, as they can also be found in 50% of women with benign disorders. Testosterone and DHEAS levels below these thresholds, however, essentially exclude an androgen-secreting tumor. For women with abnormally elevated testosterone levels and normal DHEAS levels, a search for an ovarian tumor is begun

with ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI) of the pelvis. If both testosterone and DHEAS levels are abnormally elevated, then a radiologic evaluation of the adrenal glands is performed with MRI or CT.

To exclude the possibility of Cushing's syndrome, a 24-hour urine specimen is collected and submitted for free cortisol determination. A urine free cortisol concentration of greater than 275 nmol per day (100 μ g per 24 hours) is considered abnormal. Alternatively, the patient may be given an overnight dexamethasone suppression test in which 1 mg of dexamethasone (2 mg for obese women) is given orally at 11 PM, with a serum cortisol level measured at 8 AM the next morning. Normally, dexamethasone will suppress cortisol values to less than 140 nmol per liter (5 μ g per dl). Women having a nonsuppressed serum cortisol level or abnormal urinary free cortisol concentrations are referred for complete endocrinologic evaluation. To evaluate for 21-hydroxylase-deficient nonclassic congenital adrenal hyperplasia, a morning plasma 17 α -hydroxyprogesterone level is measured. A 17 α -hydroxyprogesterone level of 6.0 nmol per liter (2.0 ng per ml) or lower rules out this adrenal gland deficiency.⁸ A women with a 17 α -hydroxyprogesterone level above 6.0 nmol per liter should be referred for adrenocorticotrophic hormone (ACTH; corticotropin)—stimulated testing.

The diagnosis of the polycystic ovary syndrome can be confirmed with laboratory determinations of serum testosterone, DHEAS, LH, and FSH levels. In the polycystic ovarian syndrome, the total testosterone level is modestly elevated in 40% to 60% of patients. In women found to have normal testosterone levels, the levels of free testosterone will usually be elevated. The DHEAS level is normal or occasionally slightly elevated, and the LH:FSH ratio is usually greater than 2.5. Pelvic ultrasonography is not required unless an ovarian mass is found on pelvic examination; however, in obese women in whom the pelvic examination is difficult, an ultrasonogram may be helpful. Enlarged polycystic ovaries are found infrequently and usually in women who manifest other features of the polycystic ovary syndrome, such as obesity, oligomenorrhea, or amenorrhea.

Treatment

Mechanical methods of hair removal are an effective adjunct for the control of hirsutism and may be the most appropriate therapy for women with limited areas of unwanted hair growth. For women with widespread hirsutism, combining medical therapy with shaving, plucking, waxing, or electrolysis can result in a more rapid response. In addition, women who have previously abandoned these physical methods may wish to resume these measures after medical therapy has slowed the rate of new hair growth. The most common adverse effects are skin irritation (bleaching, chemical depilatories), pitting or scarring (electrolysis), or folliculitis (plucking, waxing). Shaving is considered the safest method of temporary hair removal and, contrary to popular belief,

once hair is shaved, it does not return in a darker or thicker state.

For women found to have an ovarian or adrenal tumor, Cushing's syndrome, or 21-hydroxylase-deficient non-classic congenital adrenal hyperplasia, appropriate referral should be made for specific therapy. Most women's hirsutism will be caused by the polycystic ovary syndrome or idiopathic causes, and medical therapy can be offered. Treatment is more effective at preventing further hair growth than reversing long-established hirsutism, and therefore, women with the recent onset of hirsutism are more likely to respond to treatment than those with long-standing hirsutism. In addition, because hair growth is inherently a slow process, patients are advised that an observable response may not be evident in the first three to six months. Finally, determining a therapeutic response is often difficult. Although the Ferriman-Gallwey score is useful, it is often cumbersome and hampered by the concomitant use of mechanical methods of hair removal. A patient's subjective evaluation of her hair growth may be the only measure of successful therapy, with often the most useful measure of success being a reduction in the time a woman spends removing unwanted hair.

Medical therapy for hirsutism may be directed at several levels (Table 2). Drugs can suppress ovarian or adrenal androgen secretion or block the action of androgens in target hair follicles. If a hormone abnormality has been determined, then therapy is directed at reversing that abnormality. For example, a woman with an elevated testosterone level is given drugs that suppress ovarian androgen production. Women with normal testosterone concentrations are presumed to have an increased end-organ sensitivity to androgens and therefore are more likely to respond to drugs that antagonize the tissue effects of androgens.

Suppressing Ovarian Androgen Production

Oral contraceptives are the most commonly used method to suppress ovarian androgen production. Progestins inhibit gonadotropin secretion, thereby reducing ovarian-stimulated androgen secretion. The estrogen component stimulates the hepatic synthesis of SHBG, which binds circulating testosterone, and lowers the amount of free testosterone available to hair follicles. This is especially helpful in women with the polycystic ovary syndrome who often have lower SHBG levels.

When prescribing oral contraceptives, clinicians must be aware of the possible androgenic activity of the progestational component. 19-Nortestosterone derivatives, such as norgestrel, norethindrone, levonorgestrel, and to a lesser extent, ethynodiol diacetate and lynestrenol, show partial androgen activity, especially at higher doses.⁸ The dose of these progestins should be kept to a minimum. The use of a combination of ethinyl estradiol (35 µg) and ethynodiol diacetate (1 mg) found in Demulen 1/35 has often been recommended for this reason. Newer progestins, such as desogestrel, gestogen, and norgestimate, have no important androgenic activity

TABLE 2.—Medical Management of Hirsutism

Diagnosis	Medication (Proprietary Name)
Ovarian androgen suppression	Oral contraceptives GnRH agonist Depot leuprolide (Lupron Depot) Nafarelin acetate (Synarel) Cyproterone acetate* (Androcur, Diane)
Adrenal androgen suppression	Glucocorticoids
5 α -Reductase inhibitor†	Finasteride (Proscar)
Antiandrogens	Spirolactone (Aldactone) Cyproterone acetate* (Androcur, Diane)
GnRH = gonadotropin-releasing hormone	
*Not available in the United States.	
†Inhibition of testosterone to dihydrotestosterone.	

and should be advantageous in treating hirsute women. Oral contraceptives combining low-dose estrogen and these nonandrogenic progestins include Desogen, Ortho-Cept, Ortho-Cyclen, and Ortho Tri-Cyclen. Although these oral contraceptives offer a theoretic advantage, comparisons of different oral contraceptives with regards to their effects on hirsutism are lacking.

The response to oral contraceptive use varies, but it is more likely to occur in women with elevated testosterone levels. The efficacy of oral contraceptive use for the treatment of hirsutism has recently been questioned.⁹ It may be that a few hirsute women will have substantial improvement with oral contraceptive treatment alone. Until further studies are available, oral contraceptives continue to be a first-line treatment for the majority of women with hirsutism. These hormones have the added benefits of regulating menses and ensuring contraception in women taking antiandrogens.

Gonadotropin-releasing hormone (GnRH) agonists used long term can suppress ovarian androgen production in hirsute women. The long-term use of these agonists alone, however, often leads to hypoestrogenic side effects such as hot flashes, osteoporosis, and urogenital atrophy. Therefore, they are combined with oral contraceptives or conjugated estrogens and progestins in the treatment of hirsutism.^{10,11} The GnRH agonists used in the treatment of hirsutism include nafarelin acetate as an intranasal spray given 400 µg twice a day, or depot leuprolide acetate, 3.75 mg monthly. The use of GnRH agonists for the treatment of hirsutism is severely limited by cost, and therefore therapy with GnRH agonists should be reserved for moderate to severe hirsutism that is unresponsive to oral contraceptive alone or combined with antiandrogens.

Adrenal Androgen Suppression

Glucocorticoids are used to suppress ACTH-stimulated adrenal androgen production and therefore are useful for patients with the nonclassic form of congenital adrenal hyperplasia. Treatment may be initiated with dexamethasone, 0.25 to 0.5 mg; prednisone, 2.5 to 5.0

mg; or hydrocortisone, 10 to 20 mg. Glucocorticoids are administered in the evening or at bedtime to suppress the nightly ACTH surge.

5 α -Reductase Inhibitors

Finasteride is the only 5 α -reductase inhibitor available in the United States. Finasteride blocks the conversion of testosterone to dihydrotestosterone, thereby inhibiting the androgen effects on the hair follicle. Finasteride has been approved by the Food and Drug Administration (FDA) for the treatment of benign prostatic hypertrophy, but not for hirsutism. Studies have compared the use of finasteride with that of spironolactone (an antiandrogen) and found them to have equal effectiveness in the treatment of hirsutism.¹² The use of both finasteride and spironolactone decreased terminal hair diameters by 14% and improved the Ferriman-Gallwey scores by 11%. Finasteride use is well tolerated, but studies of animals have reported ambiguous genitalia in male offspring of treated females, and therefore finasteride should not be used during pregnancy and should be combined with a reliable method of contraception, such as oral contraceptives.

Antiandrogens

One of the most useful group of medications for the treatment of hirsutism are the antiandrogens. These drugs competitively inhibit the binding of testosterone and dihydrotestosterone to their respective receptors. The most commonly used antiandrogen is spironolactone, which is an aldosterone antagonist commonly used as a potassium-sparing diuretic. Spironolactone has the additional property of competitively binding to dihydrotestosterone receptors, thus limiting the response of tissues to endogenous androgens. Although not approved by the FDA for the treatment of hirsutism, several studies have shown it to be an effective drug for this indica-

tion.^{13,14} Therapy is started with a dosage of 50 mg twice a day, which is then increased to 100 mg twice a day if no response is seen after three months. Half to three quarters of women will have a noticeable improvement with spironolactone. Adverse effects are more common at higher doses and include irregular menses (25%), gastrointestinal cramping, diarrhea, nausea, lethargy, and headache. Hyperkalemia may occur, especially in elderly women, in women with diabetes mellitus, or if taken with other drugs that may increase serum potassium levels (angiotensin-converting enzyme inhibitors). Therefore, periodic monitoring of serum potassium levels is recommended. Spironolactone should not be used in pregnancy, and appropriate contraception must be recommended. Combinations of oral contraceptives and spironolactone are, therefore, commonly prescribed for the treatment of hirsutism, to ensure contraception, and to regulate the menses.

Flutamide is a potent, nonsteroidal, selective antiandrogen without progestational or estrogenic activity that has been effective in the treatment of hirsutism.⁹ Flutamide is given at a dosage of 250 mg twice a day combined with an estrogen-containing oral contraceptive. Adverse effects include dry skin and increased appetite. A major concern with flutamide is a potentially fatal drug-induced hepatitis that occurs in 0.5% of patients given this drug. Flutamide use can also cause ambiguous genitalia in male offspring, and therefore, contraception must be maintained. Its use is not recommended for the routine treatment of hirsutism due to its expense and possible toxic side effects.

Cyproterone acetate has properties of both a potent progestin and a moderately potent antiandrogen. Cyproterone acetate, therefore, acts at two levels in the pathophysiologic process of hirsutism. It competitively inhibits the binding of dihydrotestosterone to its cytoplasmic receptor and also inhibits gonadotropin secre-

TABLE 3.—Guidelines for the Treatment of Hirsutism

Diagnosis	Initial Therapy, Dosage*	Alternative Therapy, Dosage*
Polycystic ovary syndrome	Oral contraceptives (Demulen 1/35, Desogen, Ortho-Cept, Ortho-Cyclen, or Ortho Tri-Cyclen) Spironolactone (Aldactone), 50 to 100 mg bid	Finasteride (Proscar), 5 mg qd GnRH agonist (Lupron Depot), 3.75 mg monthly
Idiopathic	Oral contraceptives (Demulen 1/35, Desogen, Ortho-Cept, Ortho-Cyclen, or Ortho Tri-Cyclen) Spironolactone (Aldactone), 50 to 100 mg bid	Finasteride (Proscar), 5 mg qd GnRH agonist (Lupron Depot), 3.75 mg monthly
Nonclassic congenital adrenal hyperplasia	Glucocorticoids: prednisone, 5 mg qhs; or dexamethasone, 0.25 mg qhs	
Cushing's syndrome	Surgical excision of ACTH-secreting pituitary adenoma or ectopic ACTH-secreting tumor; adrenalectomy for adrenal hyperplasia	
Adrenal tumor	Surgical excision	
Ovarian tumor	Surgical excision	
Drug-induced	Discontinue medication	

ACTH = adrenocorticotropic hormone, bid = twice a day, GnRH = gonadotropin-releasing hormone, qd = daily, qhs = every night at bedtime

*Proprietary names are given in parentheses. Demulen 1/35-21 (GD Searle & Co) contains ethynodiol diacetate, 1 mg, and ethinyl estradiol, 35 μ g. Desogen (Organon Inc) contains desogestrel, 0.15 mg, and ethinyl estradiol, 0.03 mg. Ortho-Cept 21 (Ortho Pharmaceutical Corp) contains desogestrel, 0.15 mg, and ethinyl estradiol, 0.03 mg. Ortho-Cyclen 21 (Ortho Pharmaceutical Corp) contains norgestimate, 0.25 mg, and ethinyl estradiol, 0.035 mg. Ortho-Tricyclen (Ortho Pharmaceutical Corp) contains norgestimate, 0.18 mg, and ethinyl estradiol, 0.035 mg. Lupron Depot (TAP Pharmaceuticals) contains 3.75 mg of leuprolide acetate.

tion because of its progestational properties.^{15,16} An oral contraceptive using cyproterone acetate as the progestin would appear to be well suited for the treatment of hirsutism. Cyproterone acetate is not available in the United States in any form, but it is available as a single agent (Androcur) or combined with estrogen in an oral contraceptive (35 or 50 µg of ethinyl estradiol and 2 mg of cyproterone acetate) in Europe, Canada, and Mexico (see Table 2). Drug-induced hepatitis is a rare complication that may require periodic monitoring of liver enzyme levels.

Treatment Guidelines

Initial therapy for women with hirsutism due to the polycystic ovary syndrome or idiopathic causes can be started with spironolactone or oral contraceptives (Table 3). Certainly, in women with menstrual irregularities or contraceptive concerns, oral contraceptives are considered a first-line agent. Spironolactone is generally well tolerated and usually efficacious. Women who are intolerant or nonresponsive to spironolactone use may be given a trial of finasteride combined with an estrogen-containing oral contraceptive. In women with severe hirsutism who are not responsive to the use of antiandrogens, oral contraceptives, and finasteride, the use of GnRH agonists should be considered. Finally, if hirsutism continues or worsens despite these treatment modalities, the clinician should consider reevaluating for a serious underlying disorder.

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The Pharmacokinetics of Flutamide and Its Major Metabolites After a Single Oral Dose and During Chronic Treatment

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Summary. Flutamide is a nonsteroidal antiandrogen used in the treatment of prostatic carcinoma. We have investigated the disposition of flutamide and its two major metabolites in ten urological in-patients without significant liver or renal disease.

After oral administration flutamide is absorbed from the gastrointestinal tract with a t_{\max} of about 2 h.

Flutamide undergoes extensive first-pass metabolism, and its major metabolites are 2-hydroxyflutamide and the hydrolysis product 3-trifluoromethyl-4-nitroaniline.

After the oral administration of a single dose of 250 mg or 500 mg maximum flutamide plasma concentrations of 0.02 and $0.1 \mu\text{g} \cdot \text{ml}^{-1}$ respectively were observed. Maximum plasma concentrations of 2-hydroxyflutamide for the same flutamide doses were 1.3 and $2.4 \mu\text{g} \cdot \text{ml}^{-1}$ (mean of $n=2$ or $n=3$).

Steady-state concentrations of the biologically active metabolite 2-hydroxyflutamide ($0.94 \pm 0.23 \mu\text{g} \cdot \text{ml}^{-1}$, mean \pm SD, $n=5$) were found at 2–4 days after the administration of 250 mg every 8 h.

The area under the plasma concentration time curve for 2-hydroxyflutamide averaged 11.4 (10.6 and 12.1) and $24.3 (21.5-29.4, n=3) \mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}$ for 250 mg and 500 mg flutamide orally.

2-Hydroxyflutamide and 3-trifluoromethyl-4-nitroaniline were eliminated monoexponentially with half-times of 4.3–21.9 and 4.3–17.2 h ($n=5$) respectively.

Key words: flutamide, prostate carcinoma; 2-hydroxyflutamide, pharmacokinetics, antiandrogen, first-pass metabolism

Flutamide (3'-trifluoromethyl-4'-nitro-2-methyl-propionylanilide) is a nonsteroidal antiandrogen devoid of other hormonal activity [1, 2] and effective in the management of patients with advanced prostatic cancer [3–7].

In 1975 Katchen and Buxbaum [8] described the

disposition and metabolism of ^3H -flutamide after a single oral dose of 200 mg in three healthy men. Its active metabolite is the hydroxylated derivative 2-hydroxyflutamide [8, 9], which has a lower IC_{50} (half maximum inhibitory concentration) than flutamide in specific binding experiments with ^3H -testosterone as ligand in various mouse, rat, and human androgen-sensitive tissues ($0.98 \mu\text{mol} \cdot \text{l}^{-1}$ vs. $6.9 \mu\text{mol} \cdot \text{l}^{-1}$ for cytosol from human prostate cancer) [10].

Knowledge of the pharmacokinetics of flutamide and its metabolites in patients is scanty. We have therefore investigated the disposition of flutamide, 2-hydroxyflutamide, and its hydrolysis product 3-trifluoromethyl-4-nitroaniline in ten urological in-patients, five with prostatic adenoma and five with prostatic carcinoma, after the oral administration of flutamide.

Some of these results were presented at the International Workshop "Endocrine Manipulation of Prostatic Tumour Growth", Heidelberg, August 15, 1986 and at the 10th International Congress of Pharmacology, Sydney, 1987.

Patients, Materials, and Methods

Flutamide, 2-hydroxyflutamide, and 3-trifluoromethyl-4-nitroaniline were gifts of Schering Corporation (Bloomfield, NJ, USA). All other chemicals were of the purest grade and obtained from Merck (Darmstadt, FRG).

Study 1

After they had given their informed consent five urological in-patients (Table 1) took 250 mg flutamide orally after meals every 8 h for 4–5 days. Blood samples were collected every day before the morning dose.

Study 2

After they had given their informed consent five urological in-patients (Table 1) received a single oral dose of 500 mg ($n=3$) or 250 mg ($n=2$) flut-

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Table 1. Patient data

Subject	Age (years)	Weight (kg)	Smoker (±)	Creatinine clearance (ml·min ⁻¹)	LDH (U·l ⁻¹)	Total bilirubin (μmol·l ⁻¹)	Other drugs
<i>Study 1</i>							
W.K.	76	71	—	45	156	14	co-trimoxazole triamterene, hydrochlorothiazide
K.S.	69	75	—	74	132	10	co-trimoxazole, digoxin
J.G.	60	78	—	67	217	5	metamizole, spasmolytics, co-trimoxazole
H.J.	59	72	—	62	162	7	co-trimoxazole
H.W.	77	78	+	40	138	29	co-trimoxazole, spasmolytics, tilidine, tramadole, norfloxacin, benzodiazepines
Mean (±SD)	68.2 (8.5)	74.8 (3.3)		57.6 (14.5)	161 (34)	13 (10)	
<i>Study 2</i>							
H.H.	64	75	—	66	157	10	aspirin, tramadole
W.S.	71	75	—	48	262	5	paracetamol, flurazepam
H.B.	65	60	+	48	260	7	tilidine, co-trimoxazole, benzodiazepines
J.W.	82	74	+	60	216	10	—
W.G.	70	65	+	63	146	5	salicylates, pyrazolones, benzodiazepines
Mean (±SD)	70.4 (7.2)	69.8 (6.9)		57.0 (8.5)	208 (55)	7 (3)	

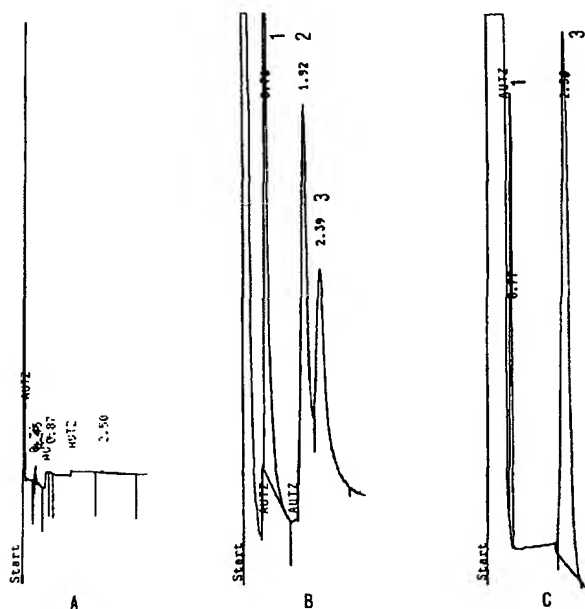


Fig. 1 A-C. Capillary gas chromatograms of 3-trifluoromethyl-4-nitroaniline (peak 1), flutamide (peak 2), and 2-hydroxyflutamide (peak 3). A: blank plasma; B: plasma spiked with flutamide, 2-hydroxyflutamide, and 3-trifluoromethyl-4-nitroaniline 250 ng·ml⁻¹ each, i.e. 25 pg onto the column; C: plasma sample 8 h after the oral administration of 250 mg flutamide

amide orally after a meal. Blood samples (10 ml) were drawn before administration and at 0.5, 1, 2, 4, 6, 8, 12, 24, 32, 40, 48, and 60 h after administration.

In both studies the blood samples were immediately centrifuged and the plasma kept frozen

(-20° C) until analyzed by a specific and rapid gas chromatographic method (Fig. 1).

Analytical Methods

100 μl of plasma were diluted with the same volume of 1 M phosphate buffer (pH 6.8). Ethyl acetate (1 ml) was added, vortex-mixed for 5 min (Eppendorf mixer 5432), and centrifuged at 8800 g (Eppendorf centrifuge 5413) for 1 min. The ethyl acetate layer was dried with approximately 50 mg anhydrous sodium sulphate and 1 μl of the supernatant was injected into the gas chromatograph (Varian GC 3700, Darmstadt, FRG). For the determination of flutamide and for plasma samples containing 50 ng·ml⁻¹ 2-hydroxyflutamide or 3-trifluoromethyl-4-nitroaniline or less the organic layer was dried at 70° C under a gentle stream of nitrogen. Toluene (100 μl) was added and 1 μl injected into the GC.

Gas chromatographic analysis was performed on a 10 m × 0.53 mm fused silica capillary column RSL 300 (polyphenylmethylsiloxane). Oven: 180° C isotherm; injection temperature: 270° C; detection: ⁶³Ni-ECD, 320° C; carrier gas: nitrogen 10 ml·min⁻¹; make-up gas: 30 ml·min⁻¹.

The detection limits for 3-trifluoromethyl-4-nitroaniline, flutamide, and 2-hydroxyflutamide were 10 ng·ml⁻¹ in each case, using 100 μl plasma. The retention times were 0.8 min (3-trifluoromethyl-4-nitroaniline), 1.9 min (flutamide), and 2.4 min (2-hydroxyflutamide). The peak areas (CI-10 printer/plotter, LDC/Milton Roy, Hasselroth, FRG)

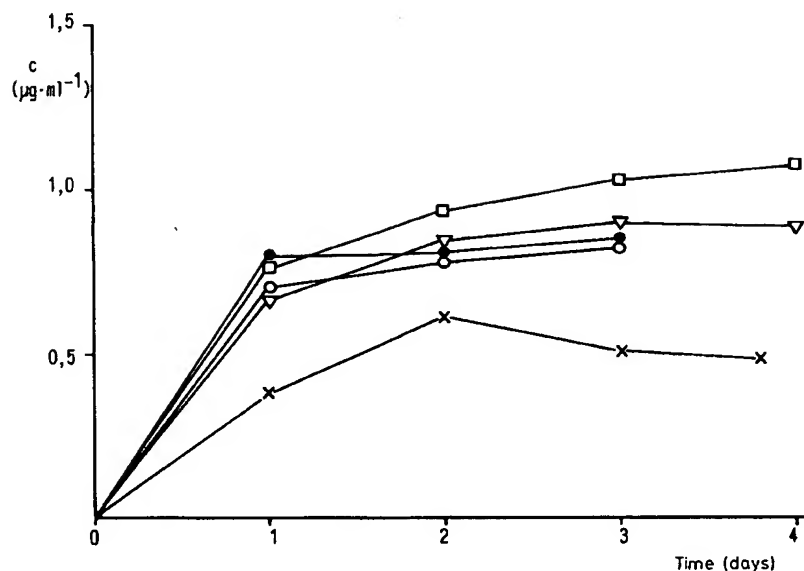


Fig. 2. Plasma concentrations (8.00 a.m.) of 2-hydroxyflutamide in five patients during repeated oral dosing with 750 mg flutamide per day (\square = H.W., ∇ = W.K., \bullet = K.S., \circ = H.J., \times = J.G.)

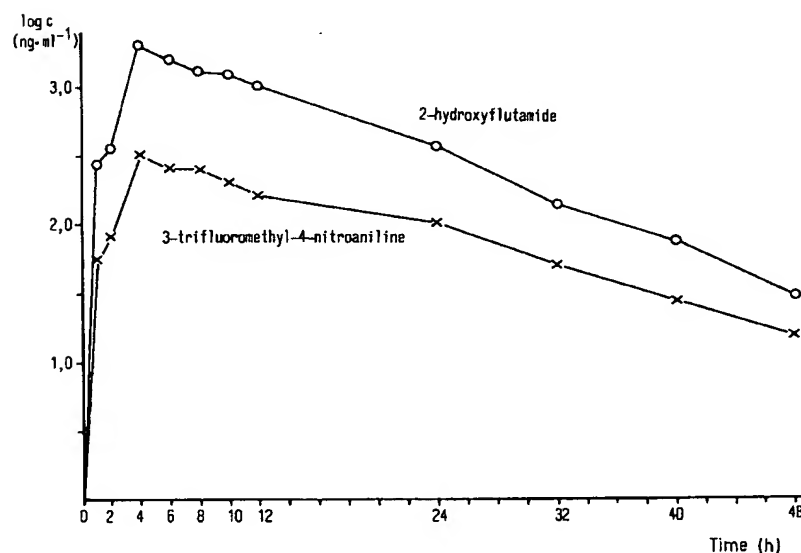


Fig. 3. Plasma concentrations of flutamide metabolites after a single oral dose of 500 mg flutamide (Patient: H.H.)

of flutamide, 2-hydroxyflutamide, and 3-trifluoromethyl-4-nitroaniline were linear in each case over a range of 0–500 pg ($r > 0.985$). Plasma concentrations of flutamide and its two metabolites were determined with a calibration curve using drug-free plasma (blood bank) spiked with 20–250 ng·ml⁻¹ flutamide, 50–2000 ng·ml⁻¹ 2-hydroxyflutamide, and 50–500 ng·ml⁻¹ 3-trifluoromethyl-4-nitroaniline ($r > 0.985$). The within-assay coefficient of variation was 7%. Recovery averaged 85%.

The area under the plasma concentration time curve (AUC) was calculated by the linear trapezoidal method. The values given in the text and tables are means with SD.

Results

Study 1

Eight hours after the oral administration of the usual clinical dose (250 mg flutamide every 8 h) no unmetabolized flutamide was detected. Steady-state concentrations of the biologically active metabolite 2-hydroxyflutamide were found after 2–4 days (Fig. 2). Steady-state concentrations of 2-hydroxyflutamide and 3-trifluoromethyl-4-nitroaniline averaged 0.94 ± 0.23 and 0.21 ± 0.09 µg·ml⁻¹ (range: 0.58–1.34 and 0.11–0.35 µg·ml⁻¹) respectively.

Table 2. Pharmacokinetics of flutamide (F), 2-hydroxyflutamide (OH-F), and 3-trifluoromethyl-4-nitroaniline (Metabolite-II) after the oral administration of 250 mg or 500 mg flutamide to five patients with prostate carcinoma

Subject (Dose, mg)	H. H. (500)	W. S. (500)	H. B. (500)	J. W. (250)	W. G. (250)
t_{\max} (h)					
F	2	1	4	1	0.5
OH-F	4	2	4	1	3.5
Metabolite-II	4	2	4	2	3.5
C_{\max} ($\mu\text{g} \cdot \text{ml}^{-1}$)					
F	0.06	0.18	0.05	0.01	0.02
OH-F	1.78	2.09	3.38	1.29	1.32
Metabolite-II	0.24	0.51	0.32	0.19	0.35
$t_{1/2}$ (h)					
OH-F	9.7	21.9	8.4	4.3	6.6
Metabolite-II	15.0	17.2	14.4	4.3	8.7
AUC ($\mu\text{g} \cdot \text{ml}^{-1} \cdot \text{h}$)					
OH-F	22.0	21.5	29.4	10.6	12.1

Study 2

After a single oral dose of flutamide, 250 mg or 500 mg, 2-hydroxyflutamide was the main component in the plasma at all measured points of time. Maximum plasma concentrations of $2.4 \pm 0.7 \mu\text{g} \cdot \text{ml}^{-1}$ (range: 1.78–3.38) and $0.1 \pm 0.06 \mu\text{g} \cdot \text{ml}^{-1}$ (range: 0.05–0.18) for 2-hydroxyflutamide and its parent compound flutamide respectively (500-mg dose) were observed after 3.3 ± 1.2 and 2.3 ± 1.5 h. The plasma concentrations of 2-hydroxyflutamide and 3-trifluoromethyl-4-nitroaniline fell monoexponentially with half-times of 4.3–21.9 h (Fig. 3, Table 2).

The area under the plasma concentration time curve for 2-hydroxyflutamide was approximately two-fold higher after the oral administration of 500 mg flutamide than after a 250 mg dose (Table 2).

Discussion

After oral administration flutamide is absorbed from the gastrointestinal tract and is mainly metabolized to the active metabolite 2-hydroxyflutamide during the first passage through the liver. Hydroxylation at this position is an uncommon metabolic process, and one would usually expect hydroxylation of the primary carbon atom or the aromatic ring. The final step in the elimination of flutamide is glucuronidation after hydroxylation of the aromatic ring [8]. During the administration of the usual dose of 250 mg flutamide three times a day, steady-state concentrations of the active metabolite 2-hydroxyflutamide were reached within 2 to 4 days at most. These experimental data agree with the theoretical values expected from the calculated half-times. The elimination half-times of 2-hydroxyflutamide and 3-

trifluoromethyl-4-nitroaniline seem to be longer after a 500-mg than after a 250-mg flutamide oral dose. However, the present data are not sufficient to decide whether the interindividual differences between the two single oral doses are statistically significant or not.

After the oral administration of a single 200-mg dose of ^3H -flutamide to three healthy men Katchen and Buxbaum found mean maximum plasma concentrations of $48 \text{ ng} \cdot \text{ml}^{-1}$ flutamide equivalents after 1–2 h. Maximum 2-hydroxyflutamide plasma concentrations of $558 \text{ ng} \cdot \text{ml}^{-1}$ flutamide equivalents, on average, were found after 2 h. Maximum plasma concentrations of 3-trifluoromethyl-4-nitroaniline of about $220 \text{ ng} \cdot \text{ml}^{-1}$ flutamide equivalents were observed after 2–4 h [8]. These data are in good agreement with the data presented here.

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5 α -Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats

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Tobias, J. H., A. Gallagher, and T. J. Chambers. 5 α -Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats. *Am. J. Physiol.* 267 (Endocrinol. Metab. 30): E853–E859, 1994.—Although androgens are thought to be important for skeletal maintenance in females and males, little is known about the mechanisms involved. To investigate this question further, we examined the effects of administering 0.01, 0.1, or 1.0 mg/kg 5 α -dihydrotestosterone (DHT) for 60 days on the skeleton of ovariectomized rats. Treatment was delayed until 90 days after ovariectomy to enable bone loss to stabilize. We found that ovariectomy markedly reduced cancellous bone volume of the proximal tibial metaphysis due to a combination of loss and thinning of trabeculae. Cancellous bone volume was partially restored by all doses of DHT, with trabecular thickness, but not number, returning to that of sham-operated animals. DHT also stimulated longitudinal bone growth and endosteal and periosteal bone formation and suppressed histomorphometric indexes of cancellous bone resorption. This suggests that DHT influences skeletal metabolism in osteopenic ovariectomized rats both by stimulating bone formation and suppressing resorption, although it is unclear which, if any, of these actions predominate at cancellous sites.

androgens; bone formation; bone resorption

HYPOGONADISM IN MEN is associated with osteopenia (9, 13) that is partially reversed by testosterone treatment (8, 13), suggesting that androgens play a physiological role in skeletal maintenance in the male. Furthermore, this role may extend to females, since serum testosterone has been found to correlate with bone mass in premenopausal women (3). In addition, investigations into the use of androgen-related anabolic steroids in the treatment of women with postmenopausal osteoporosis generally report an increase in bone mass (6, 11, 17). However, studies in both men (1, 10, 24) and women (2, 5, 6) have failed to resolve whether this reflects a predominantly anabolic or antiresorptive action.

The mechanisms by which androgens influence skeletal metabolism have been addressed using male rats, in which androgens are also important for skeletal maintenance (25, 27–29). As in clinical studies, androgens were found both to stimulate bone formation (30) and to suppress resorption (27, 28). Moreover, in a recent study, administration of the androgenic antagonist flutamide was found to cause loss of skeletal calcium from female rats, attributed to a reduction in bone formation (12). However, although this suggests that androgens are also important in regulating bone formation in female rats, no study has examined the effect of androgens on specific skeletal sites in these animals.

To investigate the mechanisms involved in androgen's action on the female skeleton, we therefore carried out a

histomorphometric assessment of the effects of androgen administration in female rats. Because the ovaries are the major source of androgens in ovulating animals, we used ovariectomized (OVX) rats, delaying treatment until 90 days after ovariectomy to enable cancellous bone volume to stabilize (30). To reduce the possibility of effects of androgen treatment being due to aromatization, we used the nonaromatizable 5 α -dihydrotestosterone (DHT; see Ref. 16).

MATERIALS AND METHODS

Female Wistar rats (13 wk old) obtained from the St. George's Hospital Medical School stock were OVX under halothane anesthesia using a dorsal approach or subjected to sham ovariectomy (sham). They were then divided into one group of seven sham animals and five groups of seven OVX animals and housed at 21°C with a 12:12-h light-dark cycle. Food (Rat-mouse Diet I; Special Diet Services, Witham, Essex, UK) and water were available ad libitum. DHT (Sigma, Poole, Dorset, UK) was dissolved in 5% benzyl alcohol (Sigma) and 95% corn oil (Sigma). DHT [0 (i.e., vehicle alone), 0.01, 0.1, or 1.0 mg/kg] was then given by daily subcutaneous injection for 60 days, delaying treatment until 90 days after ovariectomy. To provide a pretreatment baseline value for cancellous bone volume (BV/TV), one group of OVX animals was killed 90 days after ovariectomy, before the start of the 60-day treatment period. Tetracycline hydrochloride (25 mg/kg; Lederle Laboratory, Gosport, Hants, UK) and calcein (30 mg/kg; Sigma) were administered intraperitoneally 1 and 5 days before the end of the experiment, respectively. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

At the termination of the study, animals were weighed, bled by vena caval puncture under halothane anesthesia, and then killed by cervical dislocation. Uteri were subsequently removed and weighed. Success of ovariectomy was also assessed by measuring the serum concentration of 17 β -estradiol by radioimmunoassay (Coat-a-Count; Diagnostic Products, Glyn Rhonwy, Gwynedd, UK). Right tibiae were subsequently freed from soft tissue, fixed in 70% alcohol for 48 h, then dehydrated through graded alcohols and embedded without decalcification in London resin (London Resin, Basingstoke, Hants, UK). Longitudinal sections of the proximal metaphysis were prepared using a Jung K microtome (Cambridge Instruments, Cambridge, Cambs, UK); 5- μ m sections were stained with toluidine blue, and 14- μ m unstained sections were prepared for fluorescence microscopy. Bone histomorphometry was performed using transmitted and epifluorescent light microscopy linked to a computer-assisted image analyzer (Perceptive Instruments, Halstead, Essex, UK). Bone volume and surface parameters were measured by tracing relevant features with a cursor on the video screen image. All sections were examined blind.

BV/TV was assessed at the proximal tibial metaphysis on two nonconsecutive toluidine blue-stained sections for each animal. The standard area of 2.5 mm² employed, which was examined at $\times 25$ magnification, was situated 1 mm from the

Table 1. *Effect of 5 α -dihydrotestosterone on body and uterine weight and serum 17 β -estradiol*

Group	Body Wt, g	Uterine Wt, g	17 β -Estradiol, pg/ml	Final Age, days
Sham	338 \pm 12	0.61 \pm 0.06	36.8 \pm 12.3	240
OVX 90 days	393 \pm 10*	0.16 \pm 0.01*		180
DHT, mg· kg ⁻¹ ·day ⁻¹				
0	409 \pm 15*	0.12 \pm 0.01*	13.5 \pm 2.1*	240
0.01	425 \pm 15*	0.12 \pm 0.01*	12.9 \pm 2.3*	240
0.1	454 \pm 27†	0.12 \pm 0.01*	18.3 \pm 4.6*	240
1.0	425 \pm 17*	0.47 \pm 0.02‡	15.4 \pm 2.5*	240

Results show means \pm SE for body weight, uterine weight, serum 17 β -estradiol concentration, and final age of animals in sham rats and ovariectomized (OVX) animals killed after 90 days or subsequently given 5 α -dihydrotestosterone (DHT) for 60 days. * P < 0.05 vs. sham; † P < 0.05 vs. sham and 0 DHT; ‡ P < 0.05 vs. all other groups [by analysis of variance (ANOVA)].

growth plate. This distance was chosen to exclude both the primary spongiosa (maximum width 0.5 mm) and bone formed during the course of the experiment, as estimated from the product of treatment duration (60 days) and longitudinal growth rate (LGR; maximum 7.4 μ m/day). BV/TV was expressed as percentage of tissue volume composed of bone. Trabecular thickness and number were calculated according to standard assumptions (19). Static parameters [osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and osteoclast number (NOc./BS)] were recorded at \times 160 magnification in the same area as that used for BV/TV measurements.

Fluorochrome measurements were made on two nonconsecutive unstained 14- μ m-thick sections per animal. Longitudinal bone growth was obtained from the distance between the tetracycline and calcein bands lying distal to the epiphyseal growth plate, and LGR was derived by dividing this distance by the time interval between the administration of these two labels. Fluorochrome-labeled trabecular surfaces were measured at \times 160 magnification throughout a corresponding area to that used for assessing bone volume. The proportion of trabecular surface covered by double fluorochrome label (dLS/BS) was recorded, as was the mineral apposition rate (MAR), the latter being obtained by dividing the interlabel distance by the time interval between label administration. The bone formation rate (BFR/BS; tissue level, total surface referent) was obtained from the product of dLS/BS and MAR. Values for the apposition rate were not corrected for the obliquity of the plane of section of cancellous bone.

Cross sections of the tibial diaphysis, consisting of 14- μ m unstained sections taken immediately proximal to the tibiofibular anastomosis, were obtained for assessment of cortical parameters. The latter were assessed on two unstained diaphysis cross sections per animal as follows: total bone area, medullary area, cortical bone area (total bone area - medullary area), periosteal dLS/BS, MAR, and BFR/BS, and endocortical dLS/BS. Results from the histomorphometric analysis are expressed as the means \pm SE. Statistical analysis was by Fisher's least-significant difference method for multiple comparisons in a one-way analysis of variance. Tests were carried out with Statview 4.0 (Abacus Concepts, Cupertino, CA). Statistical significance was considered at P < 0.05.

RESULTS

As expected, ovariectomy led to an increase in body weight (Table 1). Body weight was also significantly greater in OVX animals treated with 0.1 mg/kg DHT

compared with OVX animals given vehicle alone. Results for uterine weight confirmed ovariectomy. In addition, 1.0 mg/kg DHT was found to increase uterine weight to an intermediate value between sham and OVX animals, consistent with a previous report that DHT exerts a uterotrophic effect at high doses (21). Serum concentration of 17 β -estradiol fell significantly after ovariectomy and was unaffected by treatment with DHT.

Ovariectomy led to a marked decrease in BV/TV (Fig. 1 and Table 2) due to a reduction in the number and thickness of trabeculae (Figs. 2, A and B). Although indexes of cancellous bone formation and resorption were not significantly different between OVX rats given vehicle alone and sham animals, our results are consistent with bone turnover remaining elevated, albeit to a modest extent, by termination of the experiment 150 days after ovariectomy (Tables 2 and 3). Age itself had little effect on cancellous bone formation and resorption, as judged by comparing these indexes in OVX animals killed 90 days after ovariectomy with those subsequently treated with vehicle for 60 days. In contrast, OVX rats showed significant age-related decreases in longitudinal growth and periosteal and endocortical bone formation (Fig. 3 and Table 4).

Treatment of OVX animals with 0.01, 0.1, and 1.0 mg/kg DHT for 60 days was associated with a greater BV/TV than OVX animals either killed 90 days after ovariectomy or subsequently treated with vehicle alone for 60 days (Fig. 1 and Table 2). This increase in BV/TV after treatment with DHT was partly caused by a dose-responsive increase in trabecular number (Fig. 2A). However, only 1.0 mg/kg DHT significantly increased trabecular number compared with the pretreatment OVX group, and, in all DHT-treated groups, trabecular number remained substantially below that of sham-operated animals. Our results also suggested that an increase in trabecular thickness had contributed to the gain in BV/TV after treatment of OVX animals with DHT (Fig. 2B). In contrast to the effect of DHT on trabecular number, all concentrations of DHT significantly increased trabecular thickness compared with the pretreatment OVX group, with this being restored, if anything, to above that of sham-operated animals.

Static and dynamic indexes of cancellous bone formation were similar in OVX animals given DHT or vehicle alone (Tables 2 and 3). However, the increase in dLS/BS and BFR/BS in OVX animals treated with 0.01 and 0.1 mg/kg DHT reached significance compared with sham animals (Table 3). Although 0.01 and 0.1 mg/kg DHT had no significant effect on cancellous bone osteoclast indexes, these were reduced by 1.0 mg/kg DHT (Oc.S/BS and NOc./BS; Table 2).

DHT was also found to cause dose-responsive stimulation of LGR (Fig. 3) and of periosteal MAR, dLS/BS, and BFR/BS (Table 4). In addition, 0.01 and 0.1 mg/kg DHT increased endosteal dLS/BS. In contrast, 1.0 mg/kg DHT suppressed endocortical dLS/BS, presumably reflecting the tendency of this dose to suppress bone turnover. Despite these effects on cortical bone formation, DHT had little effect on diaphyseal dimensions

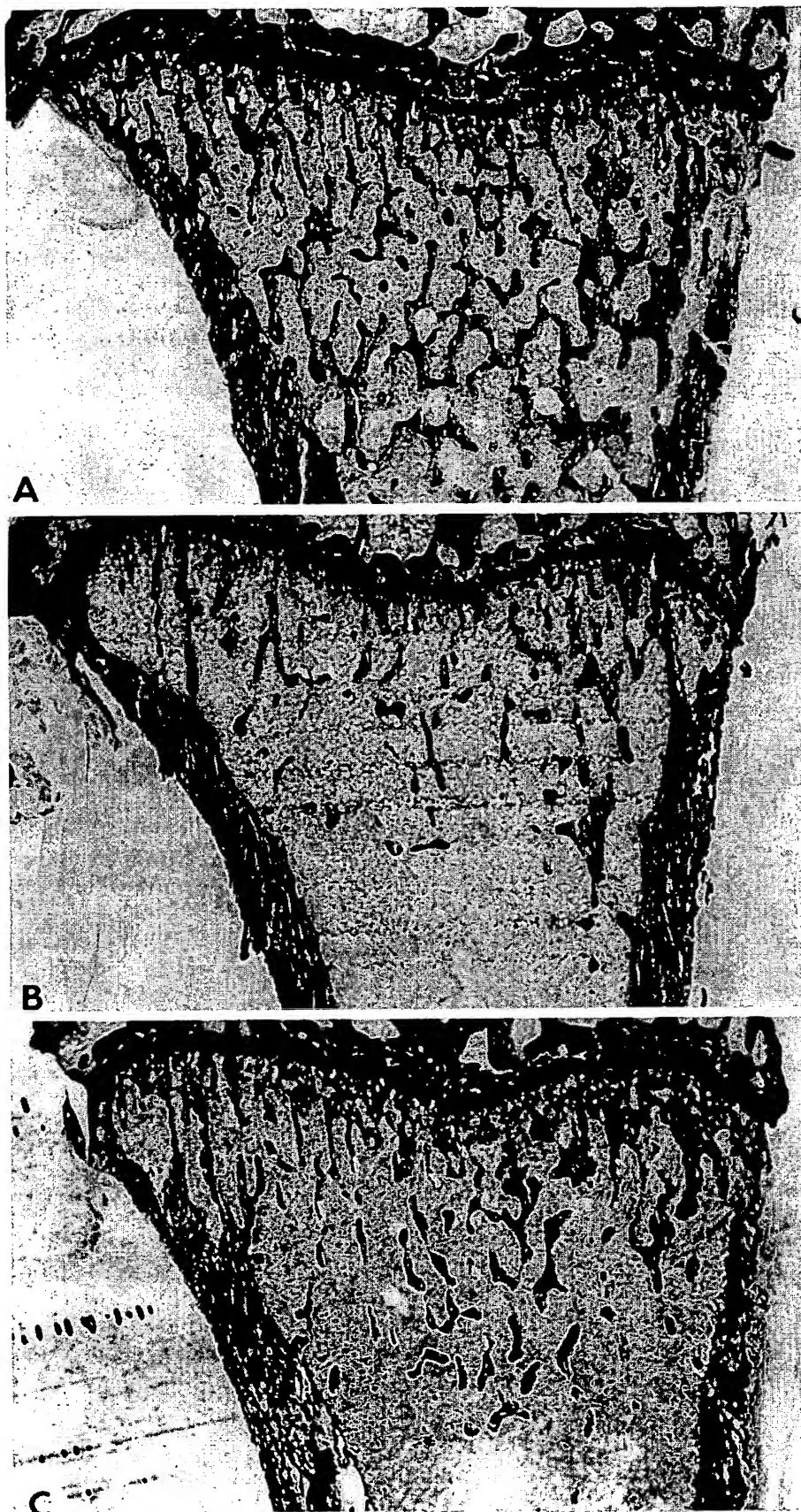


Fig. 1. Longitudinal sections of proximal tibial metaphysis stained with toluidine blue from sham rat (A), ovariectomized (OVX) rat given vehicle alone (B), and OVX rat given 1.0 mg/kg 5 α -dihydrotestosterone (C; magnification $\times 25$).

Table 2. Effect of 5 α -dihydrotestosterone on cancellous static histomorphometric parameters

Group	BV/TV, %	Ob.S/BS, %	Oc.S/BS, %	NOc./BS, n/mm
Sham	33.1 \pm 1.6	0.62 \pm 0.11	0.16 \pm 0.04	0.06 \pm 0.01
OVX 90 days	10.4 \pm 1.8*	1.37 \pm 0.39	0.23 \pm 0.04	0.10 \pm 0.02
DHT, mg· kg ⁻¹ ·day ⁻¹				
0	9.4 \pm 1.8*	1.16 \pm 0.36	0.26 \pm 0.06	0.12 \pm 0.03
0.01	15.7 \pm 2.7†	1.32 \pm 0.55	0.23 \pm 0.05	0.10 \pm 0.03
0.1	17.0 \pm 1.6†	1.09 \pm 0.20	0.29 \pm 0.06	0.12 \pm 0.03
1.0	18.8 \pm 1.2†	0.83 \pm 0.16	0.08 \pm 0.07‡	0.04 \pm 0.03§

Results show means \pm SE for cancellous bone volume (BV/TV), osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and osteoclast number (NOc./BS) at proximal tibial metaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham; † P < 0.05 vs. sham, OVX 90 days, and 0 DHT; ‡ P < 0.05 vs. OVX 90 days, 0 DHT, and 0.1 DHT; § P < 0.05 vs. 0 and 0.1 DHT (by ANOVA).

over the duration of the study (Table 5). Cross-sectional and cortical areas were found to be greater in OVX animals than in sham animals when results from OVX groups were pooled (see Table 5), consistent with previous reports of minor changes in diaphyseal area after ovariectomy in the rat (26).

DISCUSSION

We have found that DHT partially restores cancellous bone volume in female rats rendered osteopenic by ovariectomy. This increase reflected a net gain in bone volume, rather than the prevention of further bone loss, since DHT also increased bone volume compared with OVX rats killed immediately before the start of DHT administration. In previous studies of the effects of androgens in the rat, orchidectomy was noted to cause loss of cancellous bone in males (25, 27–29), which was prevented by giving androgens such as DHT (28). In addition, a microdensitometric study found that the anabolic steroid nandrolone decanoate increased bone mineral content in osteopenic OVX rats, although this effect was limited to the diaphysis (20). However, microdensitometry does not accurately distinguish trabecular from cortical bone and does not enable the histodynamic assessment of bone formation and resorption. To our knowledge, there have been no previous histomorphometric assessments of the effects of androgens in female rats.

The increase in bone volume after DHT administration appeared to be due to an increase in both trabecular number and thickness. However, the effect on trabecular thickness appeared stronger, since this was increased by all doses of DHT. Furthermore, DHT restored trabecular thickness, but not number, to that of sham-operated animals. In fact, it seems likely that agents that are able to increase cancellous bone volume of the human skeleton do so by enlarging existing trabeculae, rather than by causing their formation de novo. If so, the increase in trabecular thickness that we have found after treatment of OVX rats with androgens may accurately reflect the mechanism of increase in bone mass previously observed in women treated with

anabolic steroids (6, 11, 17). However, there have been no bone biopsy studies that address the effects of these agents on trabecular architecture in postmenopausal women.

Cancellous bone formation rate was significantly increased compared with sham rats in OVX animals treated with 0.01 and 0.1 mg/kg DHT, but not those given vehicle alone, consistent with additive effects of ovariectomy and DHT on cancellous bone formation. This suggests that DHT may have increased cancellous bone volume in osteopenic OVX rats at least in part by stimulating bone formation. However, because indexes of cancellous bone formation in vehicle- and DHT-treated OVX rats were not significantly different, it remains unclear as to whether cancellous bone forma-

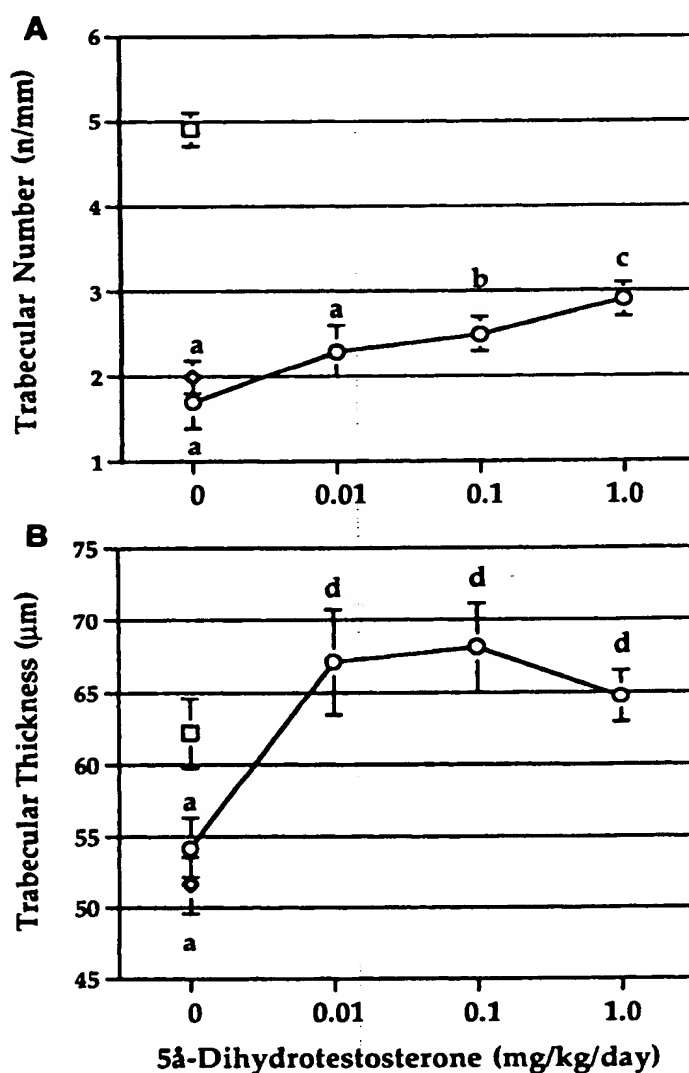


Fig. 2. Results show means \pm SE trabecular number (A) and trabecular thickness (B) at proximal tibial metaphysis in sham \square and OVX animals killed 90 days after ovariectomy (\circ ; OVX 90 days) or subsequently given 5 α -dihydrotestosterone (DHT) for 60 days (\circ). * P < 0.05 vs. sham; † P < 0.05 vs. sham and 0 DHT; ‡ P < 0.05 vs. sham, OVX 90 days, and 0 DHT; § P < 0.05 vs. OVX 90 days and 0 DHT [by analysis of variance (ANOVA)].

Table 3. Effect of 5 α -dihydrotestosterone on cancellous dynamic histomorphometric parameters

Group	dLS/BS, %	MAR, $\mu\text{m}/\text{day}$	BFR/BS, $10^{-2} \mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{day}^{-1}$
Sham	6.4 \pm 1.1	1.09 \pm 0.05	7.1 \pm 1.3
OVX 90 days	11.9 \pm 3.0	1.10 \pm 0.06	13.9 \pm 3.9
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$			
0	12.1 \pm 2.8	1.16 \pm 0.06	14.6 \pm 3.7
0.01	16.0 \pm 1.9*	1.21 \pm 0.05	19.5 \pm 2.7*
0.1	16.9 \pm 3.6*	1.22 \pm 0.03	20.9 \pm 4.9*
1.0	11.4 \pm 2.3	1.18 \pm 0.04	13.4 \pm 2.5

Results show means \pm SE of double-labeled surface (dLS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) at proximal tibial metaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham (by ANOVA).

tion was in fact stimulated by DHT. In contrast, at the growth plate, endocortical, and periosteal surfaces, a significant stimulatory effect of DHT on bone formation was observed, raising the possibility that DHT exerts an anabolic effect on the skeleton in general. Although these increases in cortical bone formation had no effect on cortical area over the study period, this may have reflected an insufficient duration of treatment for alterations in cortical, as well as cancellous, bone mass to occur.

Suppression of bone resorption may have contributed to the increase in cancellous bone volume after treatment with DHT since, although 0.01 and 0.1 mg/kg DHT had no significant effect on bone resorption, as assessed by osteoclast surface and number, 1.0 mg/kg DHT was found to reduce these resorption parameters. Interestingly, in contrast to lower concentrations, the same dose of DHT also suppressed endocortical bone formation and did not increase cancellous bone forma-

Table 4. Effect of 5 α -dihydrotestosterone on dynamic cortical histomorphometric parameters

Group	Periosteal dLS/BS, %	Periosteal MAR, $\mu\text{m}/\text{day}$	Periosteal BFR/BS, $10^{-2} \mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{day}^{-1}$	Endocortical dLS/BS, %
Sham	2.0 \pm 1.7	0.75 \pm 0.16	2.0 \pm 1.8	0.4 \pm 0.4
OVX 90 days	11.1 \pm 2.1*	0.90 \pm 0.07†	10.4 \pm 2.4*	4.1 \pm 1.9‡
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$				
0	2.8 \pm 2.0	0.51 \pm 0.04	1.3 \pm 0.8	1.5 \pm 0.8
0.01	4.6 \pm 2.9	0.76 \pm 0.14	4.4 \pm 3.0	5.5 \pm 1.5*
0.1	5.1 \pm 1.5	0.88 \pm 0.08†	4.4 \pm 1.2	4.8 \pm 1.8*
1.0	11.1 \pm 5.1*	0.89 \pm 0.07†	10.2 \pm 4.4*	0.1 \pm 0.1§

Results show means \pm SE of periosteal dLS/BS, MAR, and BFR/BS and endocortical dLS/BS at tibial diaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham and 0 DHT; † P < 0.05 vs. 0 DHT; ‡ P < 0.05 vs. sham; § P < 0.05 vs. 0.01 and 0.1 DHT (by ANOVA).

tion compared with sham animals. Therefore, inhibition of bone resorption by 1.0 mg/kg DHT may have led to a reduction in bone formation at sites where bone formation is coupled to resorption, as occurs with other inhibitors of bone resorption such as bisphosphonates, 17 β -estradiol, and calcitonin (31, 32). Hence, DHT appears to influence skeletal metabolism in osteopenic OVX rats both by stimulating bone formation and suppressing resorption, although it is unclear which, if any, of these actions predominate at cancellous sites.

These actions of DHT on skeletal metabolism are consistent with previous reports that, in male rats and humans, androgens both stimulate bone formation (1, 10, 25) and suppress bone resorption (24, 27, 28). Because DHT shows high-affinity binding with classical androgen receptors (15), which bone cells have been found to possess (7), these actions of DHT may be mediated by skeletal androgen receptors. Moreover, unlike other androgens, DHT is not thought to undergo peripheral aromatization to estrogen (16), consistent with our observation that estrogen levels were similar in OVX animals receiving DHT and vehicle alone. The actions of DHT on bone and cartilage that we and others have described are also consistent with the effects of DHT on isolated bone cell cultures. For example, DHT has

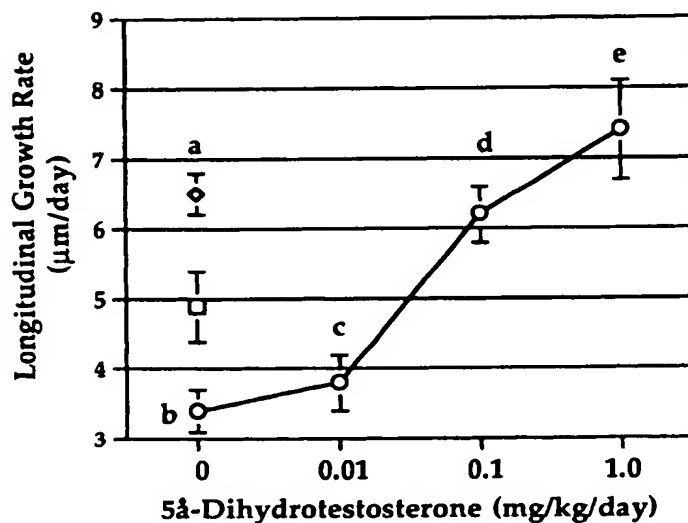


Fig. 3. Results show means \pm SE longitudinal growth rate in sham \square and OVX animals killed 90 days after ovariectomy (\circ) or subsequently given DHT for 60 days (\circ). * P < 0.05 vs. sham; ^b P < 0.05 vs. sham and OVX 90 days; ^c P < 0.05 vs. OVX 90 days; ^d P < 0.05 vs. 0 and 0.01 DHT; ^e P < 0.05 vs. sham and 0 and 0.01 DHT (by ANOVA).

Table 5. Effect of 5 α -dihydrotestosterone on cortical areas

Group	Cross-sectional Area, mm^2	Medullary Area, mm^2	Cortical Area, mm^2
Sham	4.39 \pm 0.17	0.71 \pm 0.05	3.68 \pm 0.13
OVX 90 days	4.58 \pm 0.06	0.68 \pm 0.03	3.90 \pm 0.07
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$			
0	4.79 \pm 0.06	0.76 \pm 0.06	4.03 \pm 0.08
0.01	4.90 \pm 0.18	0.83 \pm 0.04	4.07 \pm 0.17
0.1	4.79 \pm 0.25	0.77 \pm 0.08	4.02 \pm 0.18
1.0	4.76 \pm 0.11	0.76 \pm 0.05	3.99 \pm 0.12
OVX combined	4.81 \pm 0.17*	0.78 \pm 0.03	4.03 \pm 0.07*

Results show means \pm SE of cross-sectional, medullary, and cortical areas at tibial diaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. OVX combined was obtained by pooling the results from all 240-day-old OVX animals. * P < 0.05 vs. sham (unpaired 2-tailed Student's t -test).

been found to stimulate the proliferation of isolated osteoblasts (14), to increase chondrocyte DNA synthesis (4), and to suppress the bone resorptive activity of isolated osteoclasts (18).

Our findings, which suggest that relatively low doses of DHT stimulate bone formation in female rats, raise the possibility that androgens play a physiological role in regulating bone formation in females. This is consistent with the observation that the antiandrogen flutamide reduced total body calcium in female rats by suppressing bone formation (12). However, we only found an antiresorptive action of DHT at the highest dose, suggesting that androgens may not influence bone resorption in female rats under physiological conditions. In contrast, studies in orchidectomized rats suggest that physiological concentrations of androgens both stimulate bone formation and suppress resorption in male rats (25, 27). This suggests that there may be certain sex differences in responsiveness of the skeleton to androgens, which is consistent with the observation that other skeletal effects of androgens, such as stimulation of diaphyseal creatine kinase activity and DNA synthesis, are also gender specific (23).

The ability of DHT to increase cancellous bone in osteopenic OVX rats does not appear to be shared by other sex steroids such as estrogen. For example, although estrogen prevents ovariectomy-induced bone loss, it fails to significantly increase cancellous bone volume in osteopenic animals (22). These results might suggest that anabolic steroids derived from androgens are more likely to be effective at reversing bone loss in patients with postmenopausal osteoporosis. However, although androgen-related steroids have been found to increase bone mass in these patients, whether this represents an advantage over the skeletal response to estrogen therapy is currently unknown.

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Long-Term Safety of Spironolactone in Acne: Results of an 8-Year Followup Study

James C. Shaw¹ and Lucile E. White²

Abstract

Background: Spironolactone has been used for over 20 years as an antiandrogen in the treatment of acne and hirsutism. No long-term studies of the safety of spironolactone used in this manner have been published. We present a study of the long-term safety and tolerance of spironolactone in 91 women with acne who were followed for up to 8 years.

Methods: A survey questionnaire was sent to 210 patients, and a comparison chart review of all patients to whom the survey was sent was made.

Results: Ninety-one completed surveys were analyzed, comprising 506 person-years of followup and 200 person-years of spironolactone exposure. Mean treatment length was 28.5 months (range = 0.5--122 months). During the 8-year followup period, there were no cases of serious illness attributable to spironolactone use. Side effects were present in 59% and resulted in cessation of the drug in 15%. Diuretic effect and menstrual irregularities were the most common adverse effects.

Conclusions: After 200 person-years of exposure to spironolactone and 506 person-years of followup over 8 years, no serious illnesses thought to be attributed to spironolactone were reported. The long-term use of spironolactone in the treatment of acne in women appears to be safe. Side effects, however, are common, although not usually a cause for stopping the drug.

Sommaire

Antécédents: La spironolactone est utilisée depuis plus de 20 ans comme antiandrogène dans le traitement de l'acné et de l'hirsutisme. Aucune étude à long terme sur l'innocuité de la spironolactone ainsi utilisée n'a été publiée. Nous présentons ici une étude à long terme sur l'innocuité de la spironolactone et le degré de tolérance à cette substance chez 91 femmes atteintes d'acné, suivies sur une période de 8 ans.

Méthodes: Un questionnaire d'enquête a été envoyé à 210 patientes, et un sommaire comparatif des dossiers a été préparé.

Résultats: 91 questionnaires retournés ont été analysés, représentant 506 années-patients de suivi et 200 années-patients d'exposition à la spironolactone. La période moyenne de traitement était de 28,5 mois (de 0,5 mois à 122 mois). Au cours de la période de suivi de 8 ans, aucun cas de maladie grave attribuable à l'usage de la spironolactone n'a été relevé. Des effets secondaires se sont manifestés dans 59% des cas et ont disparu dans 15% des cas après l'arrêt du médicament. Les effets indésirables les plus fréquents étaient les troubles diurétiques et une irrégularité des menstruations.

Conclusions: Après 200 années-patients d'exposition à la spironolactone et 506 années-patients de suivi sur une période de plus de 8 ans, aucun cas de maladie grave qui semble causée par l'usage de la spironolactone n'a été rapporté. L'usage à long terme de la spironolactone dans le traitement de l'acné chez les femmes semble sécuritaire. Bien que

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Androgen Receptor Antagonists (Antiandrogens): Structure-Activity Relationships

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Abstract: Prostate cancer, acne, seborrhea, hirsutism, and androgenic alopecia are well recognized to depend upon an excess or increased sensitivity to androgens or to be at least sensitive to androgens. It thus seems logical to use antiandrogens as therapeutic agents to prevent androgens from binding to the androgen receptor. The two predominant naturally occurring androgens are testosterone (T) and dihydrotestosterone (DHT). DHT is the more potent androgen *in vivo* and *in vitro*. All androgen-responsive genes are activated by androgen receptor (AR) bound to either T or DHT and it is believed that AR is more transcriptionally active when bound to DHT than T. The two classes of antiandrogens, presently available, are the steroidal derivatives, all of which possess mixed agonistic and antagonistic activities, and the pure non-steroidal antiandrogens of the class of flutamide and its derivatives. The intrinsic androgenic, estrogenic and glucocorticoid activities of steroidal derivatives have limited their use in the treatment of prostate cancer. The non-steroidal flutamide and its derivatives display pure antiandrogenic activity, without exerting agonistic or any other hormonal activity. Flutamide (**89**) and its derivatives, Casodex (**108**) and Anandron (**114**), are highly effective in the treatment of prostate cancer. The combination of flutamide and Anandron with castration has shown prolongation of life in prostate cancer. Furthermore, combined androgen blockade in association with radical prostatectomy or radiotherapy are very effective in the treatment of localized prostate cancer. Such an approach certainly raises the hope of a further improvement in prostate cancer therapy. However, all antiandrogens, developed so far display moderate affinity for the androgen receptor, and thus moderate efficacy *in vitro* and *in vivo*. There is thus a need for next-generation antiandrogens, which could display an equal or even higher affinity for AR compared to the natural androgens, and at the same time maintain its pure antiandrogenic activity, and thus providing improved androgen blockade using possibly antiandrogens alone.

Introduction

Prostate cancer (PC), benign prostatic hyperplasia (BPH), acne, seborrhea, hirsutism and androgenic alopecia are well known to be sensitive to androgens [1,2] and to respond to androgen receptor antagonist (antiandrogen) therapy [3-7]. The two predominant naturally occurring androgens are testosterone (T) and dihydrotestosterone (DHT). DHT is the more potent androgen and *in vitro* expression studies have also shown that DHT is more potent in inducing transcription activation than testosterone [8]. Testosterone and DHT can, however, have some different biological functions. T-mediated functions are anabolic (muscle mass

increase, penis enlargement, scrotum enlargement and vocal cord enlargement) and spermatogenesis (male sex drive and performance), and DHT-mediated effects are increased facial and body hair, acne, scalp hair recession and prostate enlargement. All androgen-responsive genes are activated *in vitro* by AR bound to either T or DHT. AR is more transcriptionally active when bound to DHT than to testosterone.

Although castration (orchiectomy or treatment with an LHRH-agonist) causes a 90-95% reduction in serum testosterone (T) concentration [9-12], a much smaller effect is seen on the only meaningful parameter of androgenic action, namely the intraprostatic concentration of dihydrotestosterone (DHT), the most active androgen. In fact, after elimination of testicular androgens by medical or surgical castration, the intraprostatic concentration of DHT remains at

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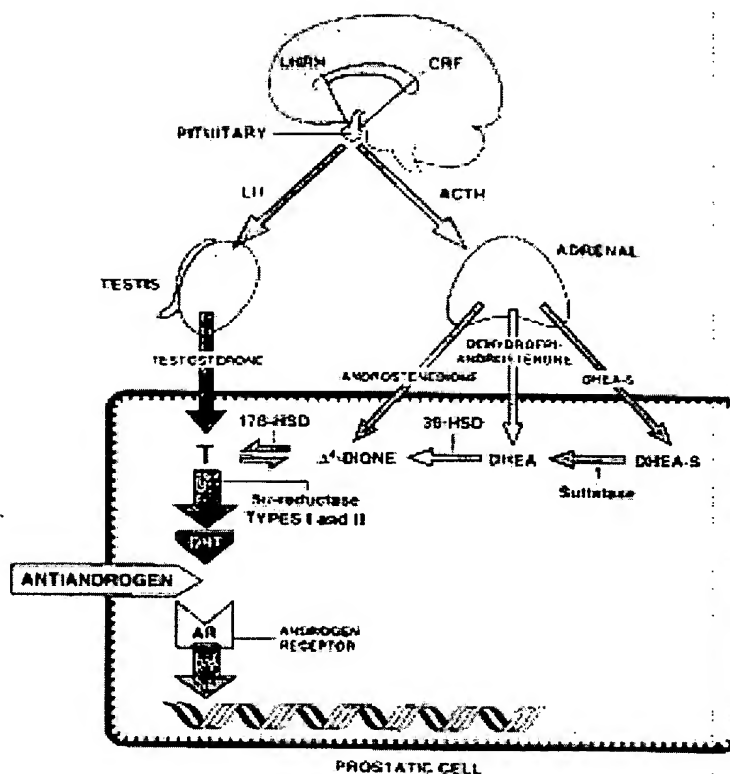


Fig. (1). Intracrine activity of the human prostate or biosynthetic steps involved in the formation of the active androgen DHT from testicular testosterone as well as from the adrenal precursors DHEA, DHEA-S and androstenedione (Δ^4 -dione) in human prostatic tissue.

approximately 40% of that measured in intact men. The importance of extratesticular androgens is also well illustrated by the finding that 40-50% of androgen metabolites remain in the circulation after castration in men [13-15]. Since recent studies show (Fig. 1) that an important proportion of androgens are produced in the peripheral tissues, including the skin and prostate, from the adrenal precursors dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S), we therefore elucidated the structure of cDNAs encoding the enzymes required for such a transformation, namely 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase [16-20], 17 β -hydroxysteroid dehydrogenase [21,22], and their corresponding genes. The structure of the cDNAs and genes encoding the two types of 5 α -reductase were also elucidated [23-26]. Since all these enzymes are expressed in the skin, prostate, and other peripheral tissues, and serum DHEA and DHEA-S are at similar levels in women and men; it is therefore not surprising that the serum concentration of the metabolites of androgens are present in women at levels 60 to 75% of those found in men of the same age.

Androgen Receptor

The androgen receptor (AR) is a member of the steroid/nuclear receptor superfamily, in which all members share basic structural and functional homology. Members of the superfamily are ligand-dependent nuclear transcription factors, and consist of three basic functional domains: the DNA binding domain, the ligand binding domain and the amino-terminal domain. However, despite the similarity in structure and function of the receptor superfamily, activation of different receptors elicits highly specific cellular responses. By studying the functional domains of receptors, and how the receptors control transcription regulation responses to different activation signals, we are beginning to understand the mechanisms controlling the specificity of receptor action. Many different naturally occurring mutations have been identified in the AR, and the study of these has allowed the localization of amino acids required for different receptor functions. These investigations, combined with *in vitro* mutagenesis studies and structural comparisons with other members of the

receptor superfamily [27,28], have allowed a greater elucidation of regions of the AR involved in ligand and DNA binding, dimerization, nuclear localization and transactivation.

AR binds ligands with high affinity, thus resulting in transformation of the receptor, associated with an increase in affinity for DNA [29,30], as a result of dissociation of heat shock proteins and a change in receptor conformation. The exact mechanisms of receptor transformation is not known: following ligand binding, the receptor changes to a more compact conformation, other conformation changes occur concomitantly with the dissociation of heat shock proteins and, then, dimerization, phosphorylation and DNA-binding occur [31].

The crystal structure of the retinoid X receptor (RXR)- α ligand binding domain has been determined by Bourguet et al. [32]. A large hydrophobic cavity is predicted to form the ligand binding pocket and evidence suggests that the same structure is present in other members of the nuclear receptor superfamily, including the AR [33,34]. This would be comprised of hydrophobic amino acids between approximately 735 and 787, and approximately 855 and 865, while the full-length receptor comprises 919 amino acids [35] (Fig. 2). Penetration of the RXR- α hydrophobic cavity by ligand requires some conformational changes of the

amino acid side chains. The ligand binding domain structures of the ligand-bound retinoic acid receptor (RAR) γ and thyroid hormone receptor (TR) suggest that the C-terminal α -amphipathic helix flips over to seal the ligand binding domain and stabilize ligand binding, exposing a novel transactivation surface [36,37].

Mechanisms of Antiandrogen Action

Since an essential step in the action of androgens in target cells is binding to the androgen receptor (Fig. 3), a logical approach for neutralizing the androgens is the use of antiandrogens or compounds which prevent the interaction of T and DHT with the androgen receptor. Since prostate cancer is so highly sensitive to androgens, the antiandrogen used should be a compound having high specificity and affinity for the androgen receptor while not possessing any androgenic, estrogenic, progestational, glucocorticoid or any other hormonal and antihormonal activity. The mechanism by which antiandrogens act may be either directly by interaction with the androgen receptor or indirectly through some nonreceptor-mediated action or metabolism or nonspecific antimetabolite activity.

The two classes of antiandrogens presently available are the steroidal derivatives, all of which possess mixed agonistic and antagonistic androgenic

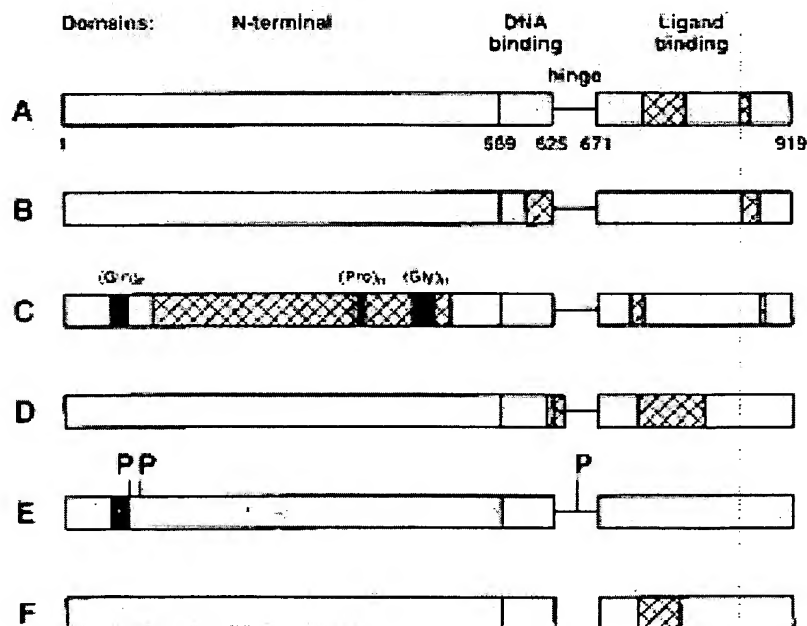


Fig. (2). Localization of AR functional regions. (A) Regions involved in the formation of the hydrophobic binding pocket. (B) Dimerization interfaces. (C) Transactivation domains. (D) Regions involved in nuclear localization. (E) Phosphorylated Ser residues. (F) Hsp90 binding site.

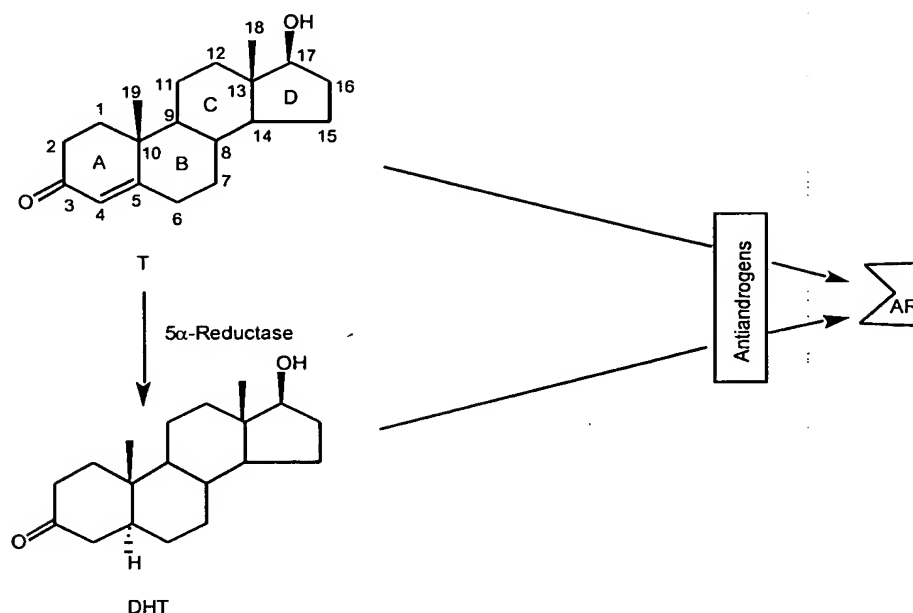


Fig. (3). Biological action of testosterone (T) and dihydrotestosterone (DHT) to androgen receptor (AR).

activities, and the non-steroidal derivatives or the pure antiandrogens of the class of flutamide, which block the androgen receptor without exerting any agonistic or any other hormonal activity. The higher efficacy of pure antiandrogens in preventing the binding of androgens to the androgen receptor has been demonstrated in a series of experimental models, including the rat ventral prostate, the level of mRNAs encoding the subunits of prostatic binding protein, ornithine decarboxylase activity as well as a growth of androgen-sensitive tumors [38-41]. As therapeutic agents, antiandrogens can be used to treat various androgen-sensitive diseases, either by topical or by systemic administration (Table 1).

Topical Treatment

Up till now, no topically active antiandrogen has been available and only some women with female

androgenization are currently treated by systemic administration of flutamide, cyproterone acetate or spironolactone [42]. For obvious reasons, such treatment cannot be applied to treat male acne and male pattern baldness [43,44]. An antiandrogen useful to treat skin disorders must be active topically and act through cutaneous androgen receptors and has to be devoid of systemic activity.

Systemic Treatment

In contrast to topical treatment, systemic administration of an antiandrogen inhibits androgenic action in all target tissues and not only at the desired target site. It thus interferes with the androgen-dependent negative feedback mechanisms regulating the secretion of androgens. The feedback action is exerted via the hypothalamo-pituitary-testicular axis. Androgens decrease the secretion of hypothalamic

Table 1. Clinical Application of Antiandrogens

Androgen effect	Application	Route of administration
Sexual behavior	Hypersexuality	Systemic
Hypothalamo-pituitary-testicular hormone (H.P.T.) secretion	Study of H.P.T. axis activity	Systemic
Bone maturation	Precocious puberty	Systemic
Sebaceous gland function	Acne, hyperseborrhea	Topical
Hair growth	Hirsutism, male pattern alopecia	Topical or systemic
Growth of androgen-dependent tumors	Androgen-dependent tumors (prostate cancer)	Systemic

LHRH and decrease the sensitivity of pituitary LH secretion to LHRH, thus decreasing the release of LH and, consequently, of testosterone secretion [45]. Suppressing androgen action at the hypothalamo-pituitary level will result in an increase in plasma LH and testosterone concentrations which can, at least partially, overcome the effect of the antiandrogen. This has been shown in the case of pure antiandrogens. For the treatment of prostate cancer, combination of chemical (LHRH agonist) or surgical castration with systemic antiandrogens have been shown to prevent the effect of the antiandrogen-induced increase in plasma testosterone [46-51].

Antiandrogens

This section will provide an overview of the development in antiandrogen structure-activity relationships (SAR) in the context of both *in vitro* potency, *in vivo* efficacy in animal models, and clinical results in the human, where available. Where the *in vitro* potency of antiandrogens is not available, SAR will be deduced from *in vivo* efficacy, although, it may not provide the real potency of antiandrogens. Since the data presented have been collected in a number of laboratories over the past few decades from a variety of tissues as sources of AR, comparison of the activity of compounds cannot be made rigorously unless they have been assayed in the same system. Correlation between receptor binding affinity and biological activity has been actively pursued to facilitate rapid and simple assessment of the characteristics of antiandrogens. One significant deficiency of measurements of receptor binding affinity is that such studies do not distinguish between agonists and antagonists. Compounds which show non-stimulatory effect on androgen-sensitive parameters such as Shionogi cells and other models should be considered as pure antiandrogens (antagonists). There are few reviews which cover partial SAR of antiandrogens [42,52,53]. In the present review, attempts are made to provide full SAR of all classes of antiandrogens, except for a few, where no SAR data are available.

Steroidal Antiandrogens

Testosterone and Dihydrotestosterone Derivatives as Antiandrogens (Table 2)

Large-scale correlation studies between the structure, binding affinities, and activities of agonists first of all led to the recognition of the structural features or combinations of features that are associated with high affinity and high activity [54,55]. The natural hormones T and 5 α -DHT, at low doses, bind

exclusively to the androgen receptor, although 5 α -DHT is a more potent competitor than T.

The dramatic decrease in the RBAs of androstanediol (4), and androstanedione (5) illustrate the need for a "17 β -hydroxy-3-one" structure for effective binding to AR. Nor-testosterone (6) displays higher affinity than its parent. Introduction of a 17 α -methyl group into T or 5 α -DHT somewhat decreases AR binding (compare 2 and 7). Combination of 17 α -methyl substitution and unsaturation leads compound RU 1881 (8) which displays high affinity for AR and PR, and also binds with GR. Methyltrienolone (RU 1881) is a highly potent androgen. The flat and flexible nature of this molecule explains its lack of specificity. Removal of the 3-keto group results in the significant loss of binding affinity of compound 9. In general, most compounds which display high affinity for the androgen receptor were androgen receptor agonists. Substituting the A- and B-ring with methyl groups decreased binding to AR and a gem-dimethyl group even further reduced the binding affinity of compound RU 2956 (10). This compound showed a mixed androgenic/antiandrogenic activity *in vivo* [56]. This initial study provided valuable information on the affinity of testosterone, dihydrotestosterone, and their derivatives and, moreover, on the functional groups which are responsible for high affinity, and the substituents and their positions, which decrease binding affinity.

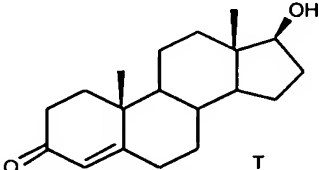
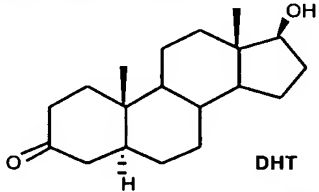
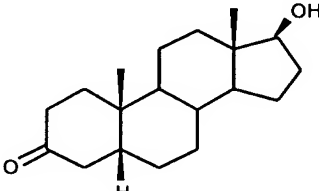
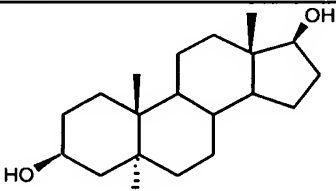
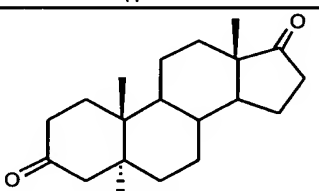
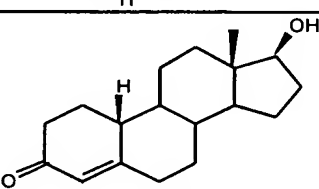
WIN17665 (11) and SH434 (12) displayed no significant antiandrogenic activity *in vivo* [58-61]. Topical application of 17 α -propyltestosterone (WIN 17665) gave a dose-related regression of the hamster flank organ and sebaceous gland size. 17 α -Propylmesterolone (SH 434), another compound of this class, reduced both sebaceous gland size and sebogenesis significantly in a dose-dependent manner. Both compounds did not show any change in prostate weight and, thus, possess little or no systemic activity on topical administration. SH 434 has been shown to be effective in acne patients [75]. Other modifications in the testosterone skeleton, for instance, introduction of gem-dimethyl group at the 16-position, gave topically active antiandrogens 14-16. However, compounds 13-19, when given subcutaneously, showed very little effect on reduction of prostate weight. Only moderate *in vivo* potency of all compounds suggests that these antiandrogens have an insufficient *in vivo* half-life and/or activity.

The well-known aldosterone antagonist (spironolactone) also displayed antiandrogenic activity in the rat and human when compared to flutamide after 6 months of therapy [42]. Spironolactone (20) reduced hirsutism score by 30% whereas, for flutamide, this

score completely decreased to normal. Moreover, spironolactone caused only a 50% reduction in acne and seborrhea (no effect on hair loss) after 3 months of therapy. In the same randomized study, flutamide caused an 80% decrease in the above scores, including hair loss. A number of other modifications in

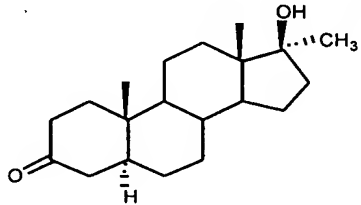
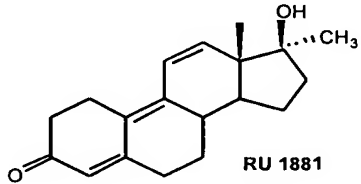
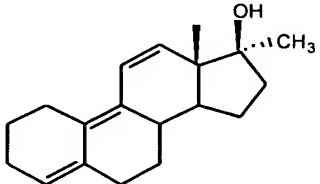
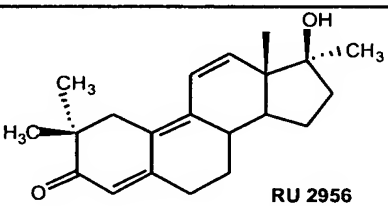
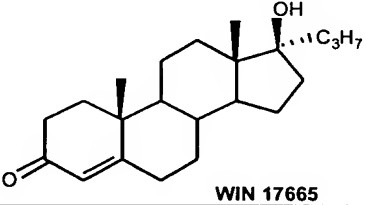
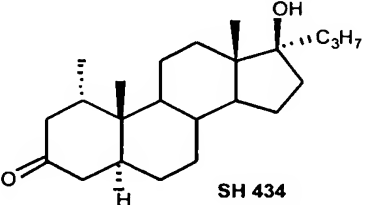
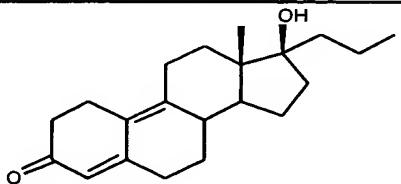
the structure of spironolactone gave other antiandrogens. For instance, lactone **24** reduced the ventral prostate weight in the rat by 39% at the oral dose of 5 mg per day while subcutaneous administration (3 mg/kg) of compound **21** reduced prostate size by 72%.

Table 2. Testosterone and Dihydrotestosterone Derivatives as Antiandrogens

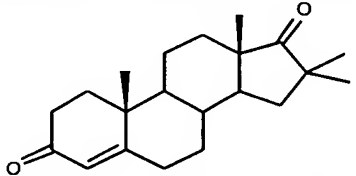
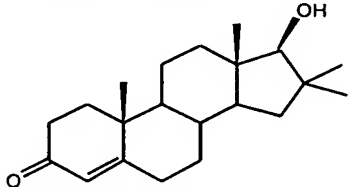
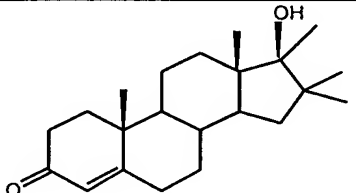
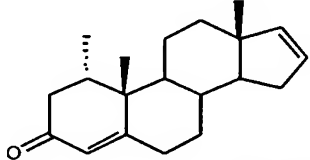
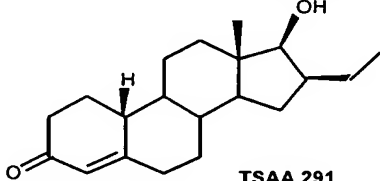
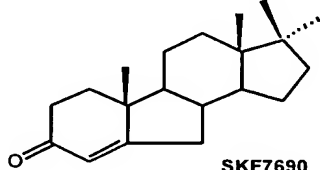
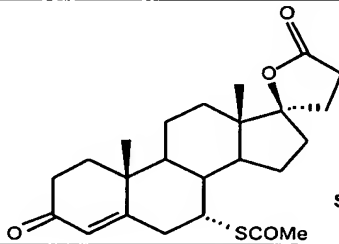
No	Structure	Androgenic/antiandrogenic activity			Ref.
		RBA	Shio. cell proli.(IC ₅₀ , nM)	% Red. of VP wt (mg/kg/d)	
1	 T	100 19/97% inh. of rAR @ 0.001/ 1 μM^a	-	-	[53,54,57]
2	 DHT	120 70/100% inh. of rAR @ 0.001/ 1 μM^a	-	-	[53,54,57]
3		5-10	-	-	[53,54,57]
4		10-15	-	-	[53,54,57]
5		1-5	-	-	[53,54,57]
6		150-200	-	-	[53,54,57]

^aSingh SM, Labrie F et al. (1998) Unpublished results.

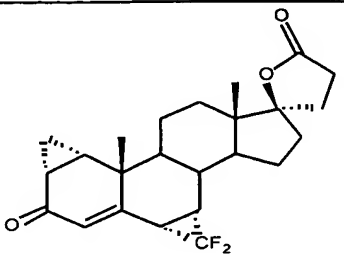
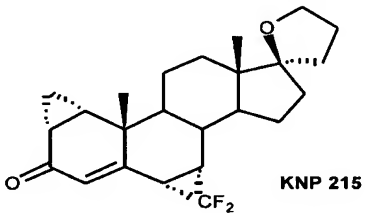
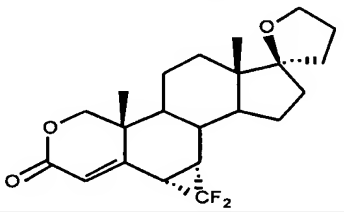
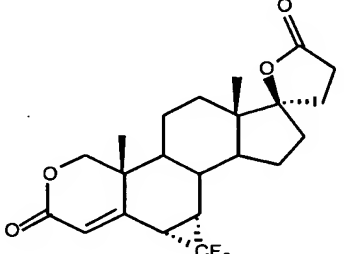
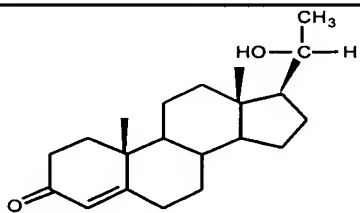
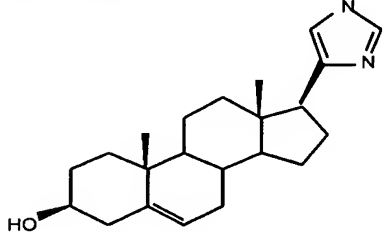
(Table 2). contd.....

No	Structure	Androgenic/antiandrogenic activity			Ref.
		RBA	Shio. cell proli.(IC ₅₀ , nM)	% Red. of VP wt (mg/kg/d)	
7		106	-	-	[53,54,57]
8	 RU 1881	200-300 70/99% inh. of rAR @ 0.001/1 μm^2	-	-	[53,54,57]
9		< 1	-	-	[53,54,57]
10	 RU 2956	50-75	-	-	[53,54,56, 57]
11	 WIN 17665	-	-	49% dec. in seb. g. @ 200 $\mu\text{g}/\text{ham./3 wk}$	[58-60]
12	 SH 434	-	-	63% dec. in seb. g. @ 5	[60,61]
13		5.7	-	19 @ 750, po	[62]

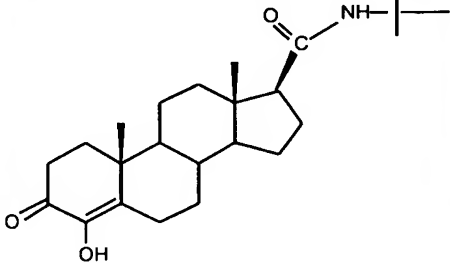
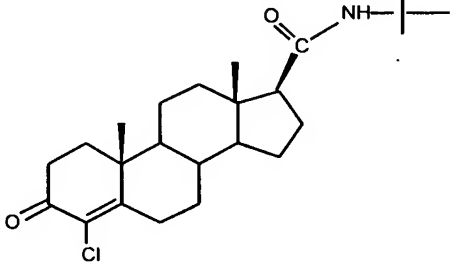
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No	Structure	Androgenic/antiandrogenic activity			Ref.
		RBA	Shio. cell proli.(IC ₅₀ , nM)	% Red. of VP wt (mg/kg/d)	
14		-	-	-	[63]
15		-	-	-	[63]
16		-	-	-	[63]
17	 GBR 21162	-	-	-	[64]
18	 TSAA 291	-	-	58 @ 2.4, rat, sc	[65,66]
19	 SKF7690	-	-	38 @ 200, sc	[67-69]
20	 Spironolactone SCOMe	-	-	33 @ 3, rat, po	[70]

(Table 2). contd.....

No	Structure	Androgenic/antiandrogenic activity			Ref.
		RBA	Shio. cell proli.(IC ₅₀ , nM)	% Red. of VP wt (mg/kg/d)	
21		-	-	72 @ 3, sc	[71]
22	 KNP 215	-	-	51 @ 10, po	[72]
23		-	-	54 @ 10, po	[72]
24		-	-	39 @ 5, po	[72]
25		-	>50% h WT AR inh. @ 0.1 μ M	-	[73]
26		-	46% h WT AR inh. @ 1 μ M 50% LNCaP AR inh. @ 1 μ M	-	[73]

(Table 2). contd.....

No	Structure	Androgenic/antiandrogenic activity			Ref.
		RBA	Shio. cell proli.(IC ₅₀ , nM)	% Red. of VP wt (mg/kg/d)	
27		-	242	-	[74]
28		-	170	-	[74]

Compounds **25** and **26**, primarily developed as inhibitors of human 17(-hydroxylase/C₁₇₋₂₀ lyase, also displayed the antagonist and agonist effects on the wild-type human AR and on the mutant human AR present in LNCaP cells [76]. Compound **26** displayed a 46% reduction in transcriptional activity at 1 μ M and more than 50% reduction of activity of the wild-type AR was obtained by compound **25** at 0.1 μ M. However, only compound **26** showed antagonistic (50% at 1 μ M) effect on LNCaP AR. A variety of C4 substituted C17 *t*-butylamide steroids developed as inhibitors of 5 α -reductase also inhibited DHT-stimulated Shionogi cell proliferation. Thus, compounds **27** and **28** displayed moderate antiproliferative activity.

Synthetic Progestins as Antiandrogens (Table 3)

Synthetic progestins, primarily prepared as progestins, also showed significant antiandrogenic activities together with androgenic activities [57,77]. CPA (**29**), the 6-chloro-1,2-methylene derivative of 17 α -acetoxypregesterone, exhibited high affinity for the rat androgen receptor (RU 1881 as a reference compound; RBA=158/203). Removal of the 1,2-methylene group gave chlormadinone acetate (CDA, **30**) with increased androgen binding, especially at short incubation times. Replacement of the chlorine by a methyl (MGA, **31**) slightly decreased binding whereas

further removal of the C6 double bond (MPA, **32**) modified binding kinetics.

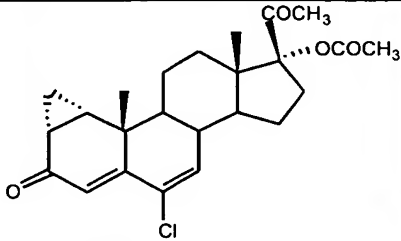
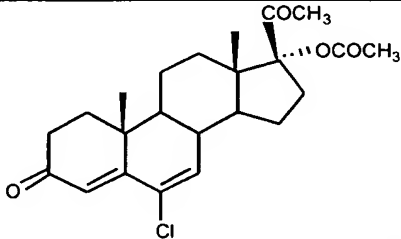
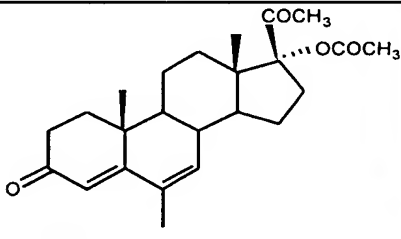
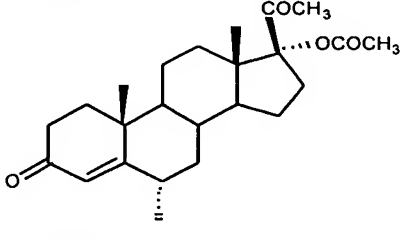
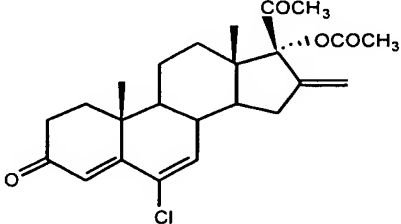
The RBA of MPA did not decrease with the incubation time, thus indicating a stronger association with AR than other synthetic progestins and explaining the relatively high androgenic activity of the compound (**32**). Other synthetic progestins **29-31** also exhibited androgenic activity on other androgen-sensitive parameters. Thus, the compounds stimulated cell growth in an androgen-sensitive clone of the mouse mammary carcinoma Shionogi SC-115 cells and their agonist activities were completely blocked by flutamide [38,39]. They also exhibited significant androgenic activity in ZR-75-1 cells co-transfected with hAR (DHT=100% at 0.1 μ M). Moreover, these progestins also exhibit glucocorticoid and antimineralocorticoid activities which seriously limit their tolerance, efficacy, and use, particularly where an optimal blockade of androgens is required, especially in prostate cancer.

Recently, other modifications in the A-, B-, C-, and D-rings of the progestin skeleton were carried out, in the hope, to obtain more potent antiandrogens. 2-Oxachlormadinone acetate (**37**) and 2-azachlormadinones (**39,40**) gave significant *in vivo* antiandrogenic activity. Thus, the potency of 2-oxachlormadinone, TPZ-4238, was the highest in the new progestin series. At the dose of 6 mg/kg/day, TPZ-4238 reduced rat ventral prostate weight by 75%. TPZ-

4238 (37), when compared with CDA, presently used in the medical management of BPH in Japan, produced a regression in canine BPH at the dosage of 0.1 mg/kg/day. This compound was 5 times more effective

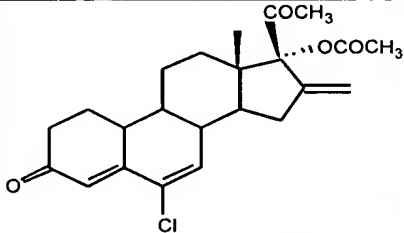
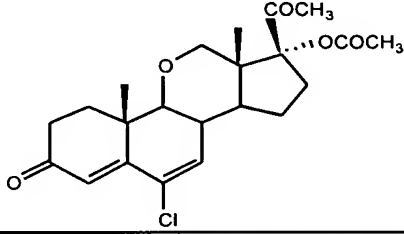
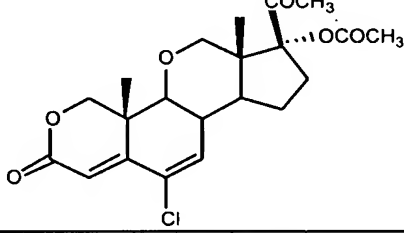
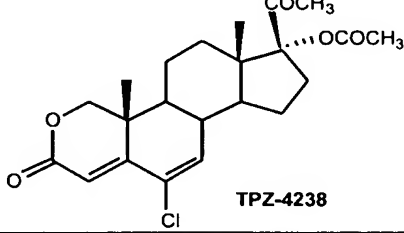
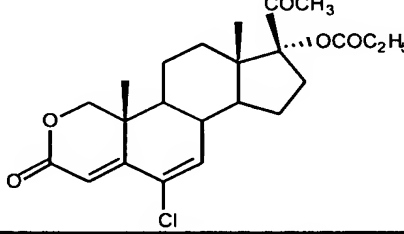
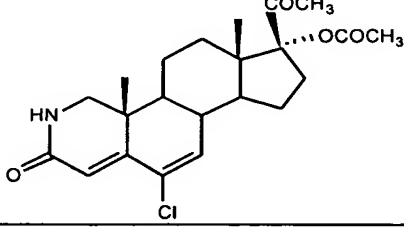
than CDA (3 mg/kg/day) [81,82]. The clinical significance of CPA and others will be discussed in the later part of the review.

Table 3. Synthetic Progestins as Antiandrogens

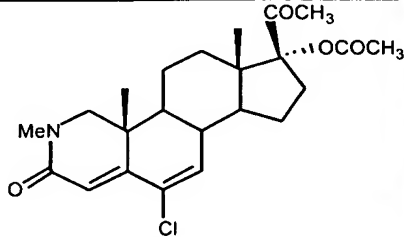
No	Structure	Antiandrogenic activity		Ref.
		RBA (30 min/2 h)	% Red. of VP wt (mg/kg/d)	
29	 <p>CPA</p>	51/16	66 @ 0.5/mice/bid, sc ^a	[38,39,57]
30	 <p>CDA</p>	81/20	20 @ 5, po 55 @ 45, po 62 @ 0.5/mice/bid/sc ^a	[38,39,57,78]
31	 <p>MGA</p>	67/19	57 @ 0.5/mice/bid/sc ^a	[38,39,57]
32	 <p>MPA</p>	40/51	47 @ 0.5/mice/bid/sc ^a	[38,39,57]
33		-	50 @ 10, sc	[79]

^aSingh SM, Labrie F et al. (1998) Unpublished results.

(Table 3). contd.....

No	Structure	Antiandrogenic activity		Ref.
		RBA (30 min/2 h)	% Red. of VP wt (mg/kg/d)	
34		-	35 @ 10, sc	[79]
35		-	12 @ 2, po	[78,80]
36		-	42 @ 2, po	[78,80]
37	 TPZ-4238	-	75 @ 6, po	[78,80-82]
38		-	79 @ 6, sc	[78,80]
39		-	57 @ 6, sc	[80,83]

(Table 3). contd.....

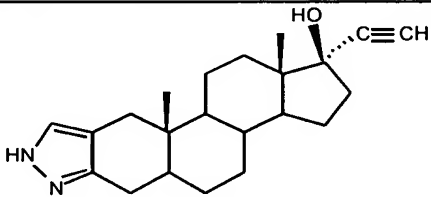
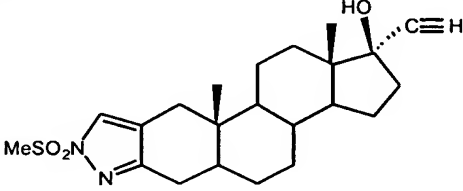
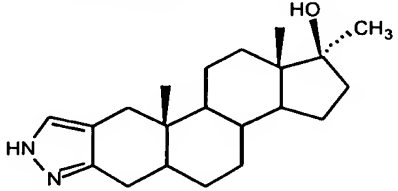
No	Structure	Antiandrogenic activity		Ref.
		RBA (30 min/2 h)	% Red. of VP wt (mg/kg/d)	
40		-	57@ 6, sc	[80,83]

Antiandrogenic Steroidal Sulfonyl Heterocycles (Table 4)

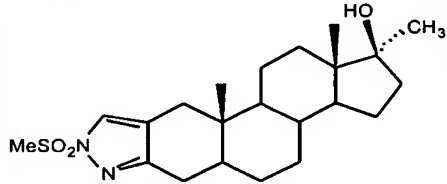
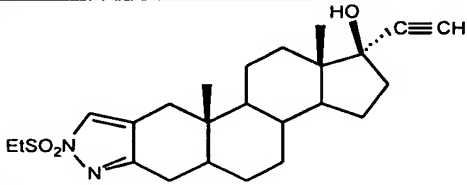
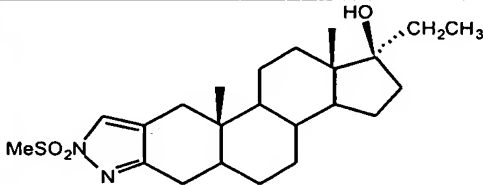
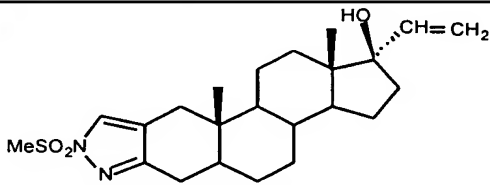
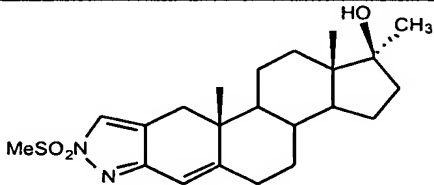
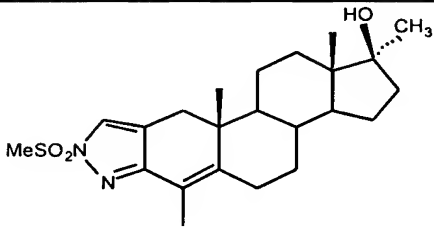
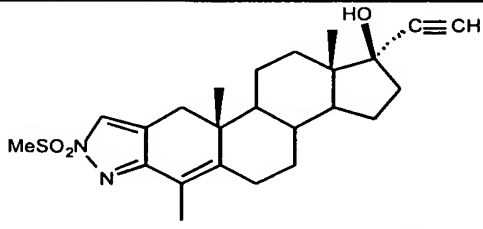
The Sterling group synthesized [84-87] and evaluated steroidal sulfonyl [3,2-c]pyrazoles and their bioisosteric sulfonyl heterocycles as androgen receptor antagonists. The parent pyrazole **41** bound strongly to the androgen receptor and displayed significant mixed androgenic/antiandrogenic activity *in vivo*. However, its 1'-methylsulfonyl derivative **42** exhibited less affinity to AR, but it was more potent than **41** *in vivo*. The 17 α -

methyl analog **43** of the parent pyrazole showed the highest affinity for AR and was a potent androgen *in vivo*. However, the 1'-methylsulfonyl derivative **44** displayed moderate affinity compared to **43**, but this compound was a potent antiandrogen *in vivo*. It thus seems that this 1'-methylsulfonyl group is critical for significant binding to AR and antiandrogenic activity. The larger alkylsulfonyl groups compared to the methyl increased the receptor affinity, and showed mixed androgenic/antiandrogenic activity (compare compounds **42** and **45**).

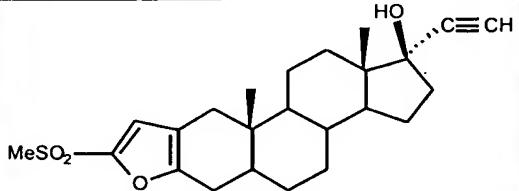
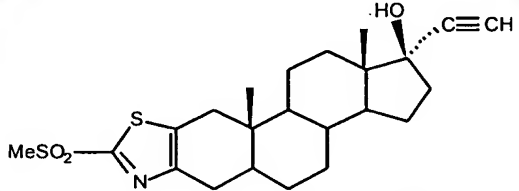
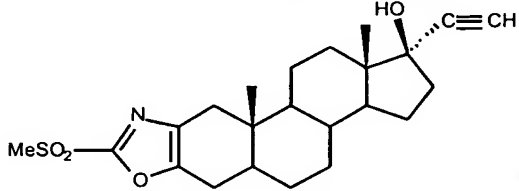
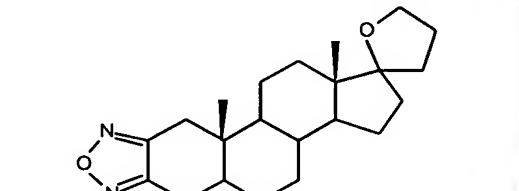
Table 4. Antiandrogenic Steroidal Sulfonyl Heterocycles

No	Structure	Antiandrogenic activity		Ref.
		RBA(1 h/18 h)	ED ₅₀ (mg/kg)	
41		28/0.8 (DHT: 87/88)	50(50% VP wt inc.)	[88]
42	 WIN 49596	2.2/0.05	15 ^a	[88,89]
43		164/0.8	-	[88]

(Table 4). contd.....

No	Structure	Antiandrogenic activity		Ref.
		RBA(1 h/18 h)	ED ₅₀ (mg/kg)	
44		16/1	10 ^a	[88]
45		2.7/0.1	100 (50% VP wt inc.)	[88]
46		3.0/0.1	41 ^a	[88]
47		4.0/0.1	33 ^a	[88]
48		12.0/0.9	16 ^a	[88]
49		18.0/1.0	3 ^a	[88]
50		7.0/0.05	14 ^a	[88]

(Table 4). contd.....

No	Structure	Antiandrogenic activity		Ref.
		RBA(1 h/18 h)	ED ₅₀ (mg/kg)	
51		1.9/0.2	8	[90,91]
52		1.4/0.16	17	[90]
53		1.2/0.05	22	[90]
54		5.8	32% @ 750 mg/kg/d	[62]

^aAndrogenic activity was not significant.

Partial and complete saturation of the 17 α -triple bond increased the affinity for AR and decreased the *in vivo* efficacy (compare compounds **42**, **46**, and **47**). In the Δ^4 -series, the binding affinity of **48** was similar to **44**. Introduction of the methyl group at the C-4 position, compound **49**, increased the affinity as well as the *in vivo* potency. A number of bioisosteric sulfonyl heterocycles were also prepared, 2'-methylsulfonyl furan **51** showing low AR affinity, but improvement of *in vivo* potency compared to **52** and **53**. Out of all, WIN 49596 (**42**) was evaluated further in a preclinical study [89]. Daily administration (20-500 mg/kg) of WIN 49596 to mature male rats for 72 days gave a significant inhibition of ventral prostate and seminal vesicle weights. At the highest dose level (500 mg/kg), the weight of the ventral prostate and seminal vesicles was reduced by 64% and 48%, respectively, without compromising reproductive function. Zanolterone (WIN 49596) is in a phase II clinical trial in human for the treatment of BPH and prostate cancer in the USA.

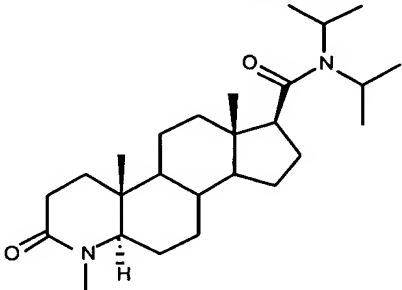
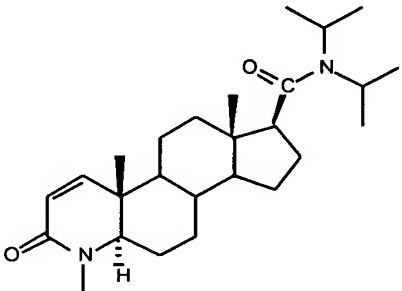
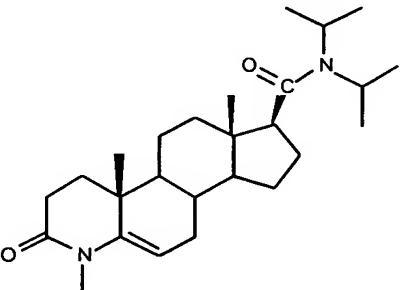
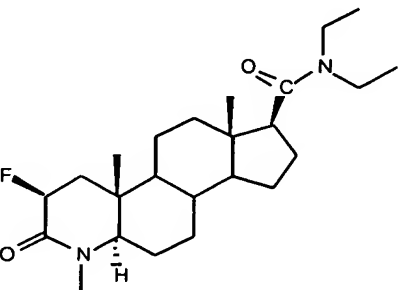
4-Azasteroids as Antiandrogens (Table 5)

4-Azasteroids primarily prepared as inhibitors of 5 α -reductase also displayed moderate to good antiandrogenic activity *in vitro* and *in vivo* [92-95]. Antiandrogenic activity varied dramatically depending upon the nature of substitution. For instance, replacement of N-CH₃ (compound **63**) by N-H (compound **62**) greatly diminished the affinity of compounds. However, introduction of the 1,2- (compare compounds **63** and **64**) or 5,6- (compare compounds **55** and **57**) double bond increased the activity relative to the parent compound. Other A-ring modifications of 4-azasteroids such as addition of 2 β -fluoro (**58**) and 1 α ,2 α -epoxy (**59**) gave enhancement in antiandrogenic activity. A range of 17-substituted azasteroids was also evaluated, N-dialkyl amides and carbonyls were less effective. On the other hand, NH-aryl amides (**63-65**) displayed very strong antiandrogenic activity. Significant loss of activity was observed when NH-aryl amide (**64**) was replaced by N-

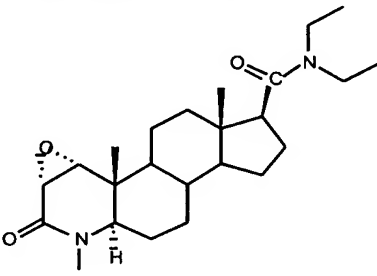
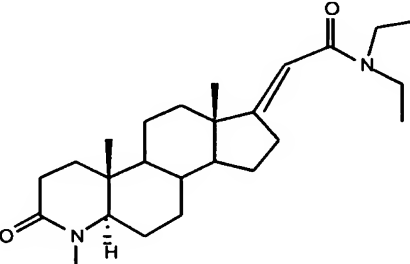
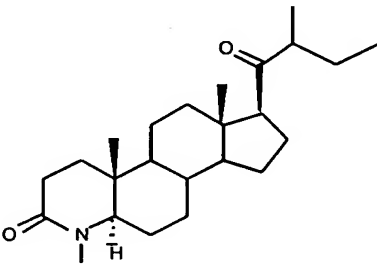
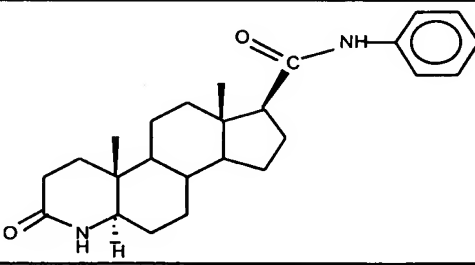
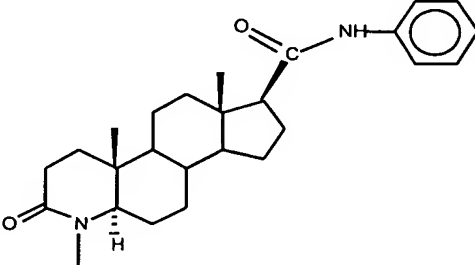
alkylaryl amide (**66**). *In vivo* assays utilizing castrated male rats, oral administration of compounds **55**, **57**, **58**, **60** and **61** in testosterone propionate treated rats caused a severe reduction of ventral prostate weight

compared to dihydrotestosterone propionate treated rats. This difference in activity versus the two androgens is due to that compounds are much more potent inhibitors of 5 α -reductase than antiandrogens.

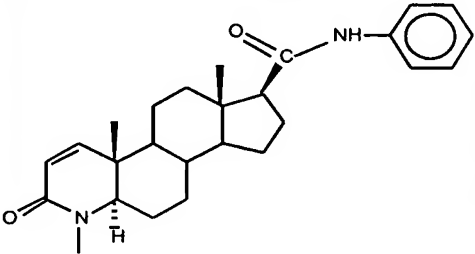
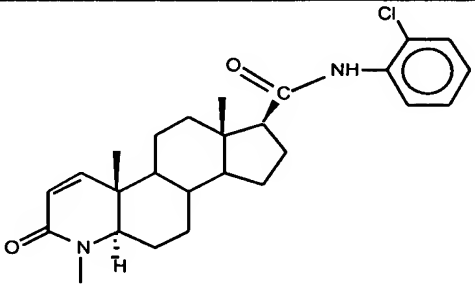
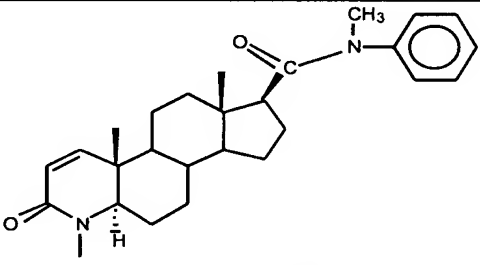
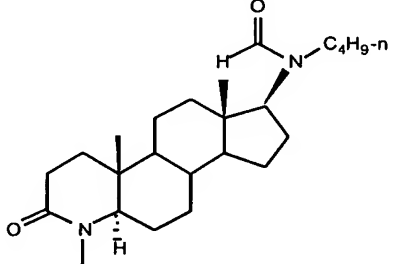
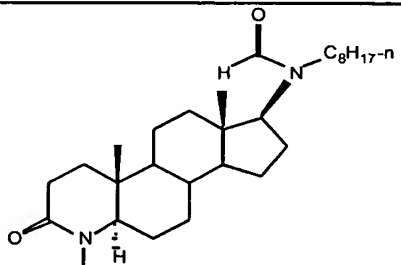
Table 5. 4-Azasteroids as Antiandrogens

No	Structure	Antiandrogenic activity			Ref.
		rAR or hAR (IC ₅₀ , nM)	Inh. of Shio.cell proli.(IC ₅₀ , nM)	% Red. of VP wt ^b (mg/kg/d)	
55		14,000(rAR)	-	>51 @ 108	[92,93]
56		10,000(rAR)	-	-	[92]
57		10,000(rAR)	-	> 25 @ 90	[92,93]
58		1800 (rAR)	-	> 23 @ 90	[92,93]

(Table 5). contd.....

No	Structure	Antiandrogenic activity			Ref.
		rAR or hAR (IC ₅₀ , nM)	Inh. of Shio.cell proli.(IC ₅₀ , nM) ^a	% Red. of VP wt(mg/kg/d) ^b	
59		710(rAR)	-	-	[92]
60		930(rAR)	-	>23 @ 108	[92,93]
61		420(rAR)	-	>25 @ 90	[92,93]
62		23,000(hAR)	-	-	[96,97]
63		90(hAR)	-	-	[96,97]

(Table 5). contd.....

No	Structure	Antiandrogenic activity			Ref.
		rAR or hAR (IC ₅₀ , nM)	Inh. of Shio.cell proli.(IC ₅₀ , nM) ^a	% Red. of VP wt(mg/kg/d) ^b	
64		5(hAR)	-	-	[96,97]
65		8(hAR)	-	-	[96,97]
66		6,000(hAR)	-	-	[96,97]
67		-	166	-	[98]
68		-	50	-	[98]

(Table 5). contd.....

No	Structure	Antiandrogenic activity			Ref.
		rAR or hAR (IC ₅₀ , nM)	Inh. of Shio.cell proli.(IC ₅₀ , nM) ^a	% Red. of VP wt(mg/kg/d) ^b	
69		-	90	-	[98]
70		-	46	-	[98]
71		-	250	-	[99]
72		-	95	-	[99]
73		-	129	-	[99]
74		-	67	-	[99]

^aAntagonism was performed on Shionogi mouse mammary carcinoma cells in the presence of DHT (0.3 nM). Reference compound: hydroxyflutamide; IC₅₀=54 nM.

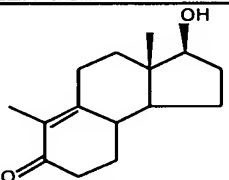
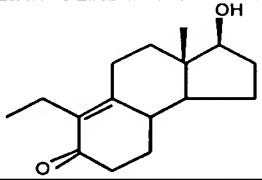
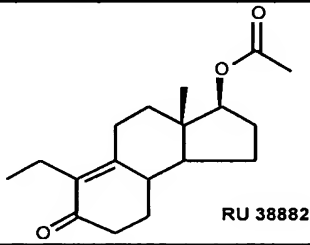
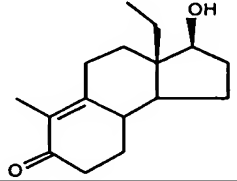
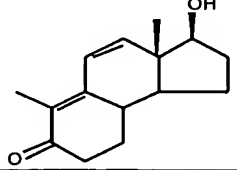
^bDihydrotestosterone propionate (0.4 mg/kg/day). Oral administration of antiandrogens.

In the C17 reversed amide class, in general, antiandrogenic activity increased as the N-alkyl chain length increased. For instance, compound **68** showed better antiproliferic activity than **67** on DHT-stimulated Shionogi cell proliferation. 17 β -Hydroxy-17 α -(ω -chloroalkyn-1'-yl)-4-azasteroids, another class of 4-azasteroids, also showed good antiandrogenic activity. In this class, introduction of the 1,2-double bond also increased the potency (compare compounds **71**, **72**, **73**, and **74**). The C4 and C5-chain lengths showed similar activity. Replacement of the chloro group by the bromo or iodo group also gave similar activity, but the corresponding hydroxy group gave inactive compounds.

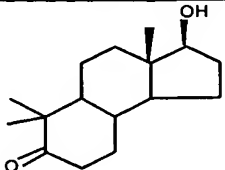
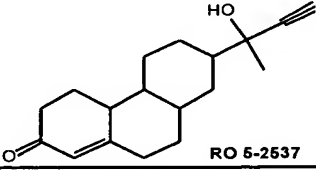
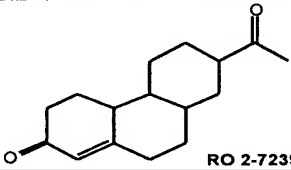
Des-A-steroidal Antiandrogens (Table 6)

In the steroid series, the presence of the 3-keto and 17 β -hydroxy groups is essential for interaction with the androgen receptor, and is important for biological activity. In the des-A-steroidal series, where the distance (8.9 Å) between the two oxygen functions (3-keto and 17 β -OH groups) is much shorter than in the steroid series (10.9 Å), the tricyclic derivatives showed noticeable affinity for the androgen receptor. A methyl group at the C10 position and conjugated double bond in the B- and C-ring enhanced the receptor affinity. When administered subcutaneously to immature castrated rats, compounds **75-79** reduced prostate weight and the most active compound was **76**.

Table 6. Des-A-steroidal Antiandrogens

No	Structure	Antiandrogenic activity		Ref
		RBA ^a	% Red. of VP wt (mg/kg/d/sc)	
75		4	27 @71 ^b	[100,101]
76		1	59 @71	[100,101]
77	 RU 38882	-	50 @71 80 @ 1/ham.(flank organ wt.)	[100-102]
78		5	10 @ 71	[100,101]
79		8	25 @ 71	[100,101]

(Table 6). contd.....

No	Structure	Antiandrogenic activity		Ref
		RBA ^a	% Red. of VP wt (mg/kg/d/sc)	
80		2	49 @ 71	[100,101]
81		-	54 @ 240 ^c	[103,104]
82		-	78 @ 17 ^d	[105]

^aRBA condition: rat prostate, 0°C, [3H]-testosterone, 0.7% ethanol, 24h.^bReference compound for *in vivo* experiment: cyproterone acetate (90% at 14 mg/kg/day). 0.7 mg/kg/day of testosterone propionate.^cTestosterone propionate (1.5 mg/kg/day).^dTestosterone. (2 mg/kg/day).

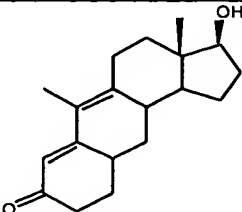
However, these compounds were weak antiandrogens compared to cyproterone acetate (**29**). Similar results were obtained when these compounds were tested locally on one hamster flank organ. Compound **77** reduced flank organ weight by 80% while under these conditions, compound **77** was more potent when compared to cyproterone acetate [102]. In another class of des-A-steroids, Ro 5-2537 showed weak antiandrogenic activity and no androgenic activity under the assay conditions used. Moreover, compound **81** also displayed progestational and uterotrophic activities. Another tricyclic derivative, compound **82**, showed potent antiandrogenic activity

along with antimyotrophic activity. Finally, this study provided valuable information that the tetracyclic structure, i.e. steroid backbone, is not essential for the androgen receptor affinity and antiandrogenic activity.

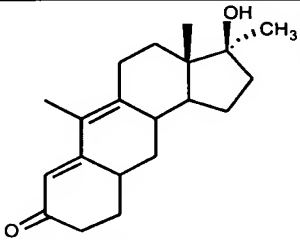
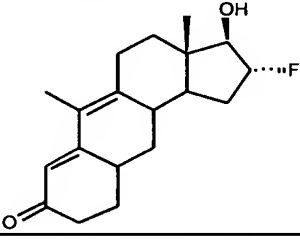
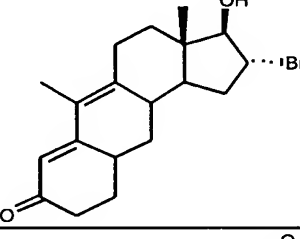
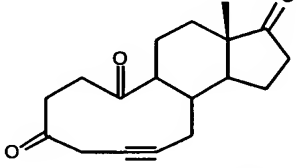
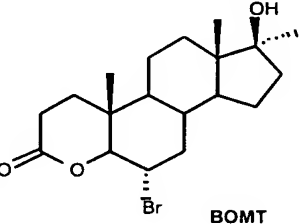
Other Steroidal Antiandrogens (Table 7)

Anthrasteroids **83-86** showed significant antiandrogenic activity *in vivo*, when administered subcutaneously. Compounds **83** and **86** also inhibited the androgen-dependent tumor growth (Shionogi-carcinoma 115) *in vivo*.

Table 7. Other Steroidal Antiandrogens

No	Structure	Antiandrogenic activity			Ref.
		RBA	% Inh. of tumor wt (mg/mice/d)	% Red. of VP wt (mg/kg/d)	
83		-	92 @ 2	43 @ 25, sc	[106]

(Table 7). contd.....

No	Structure	Antiandrogenic activity			Ref.
		RBA	% Inh. of tumor wt (mg/mice/d)	% Red. of VP wt (mg/kg/d)	
84		-	-	41 @ 25, sc	[106]
85		-	-	52 @ 25, sc	[106]
86		-	69 @ 2	-	[106]
87		≈50	-	no inh. @ 12	[107,108]
88		2.7	-	-	[109]

Non-steroidal Antiandrogens

Flutamide Derivatives as Antiandrogens (Table 8)

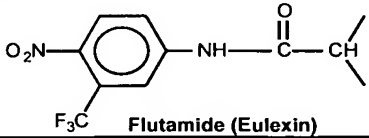
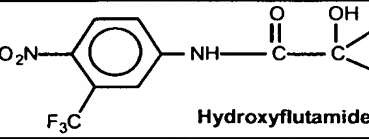
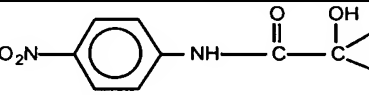
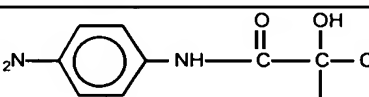
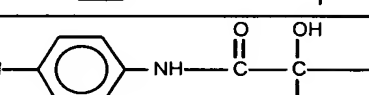
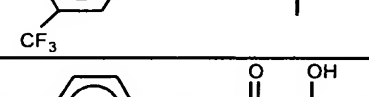
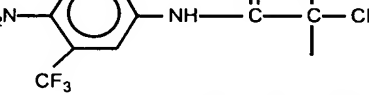
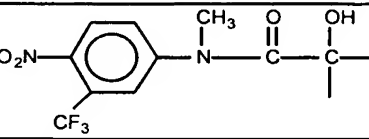
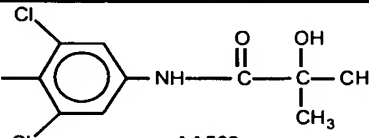
Flutamide and its derivatives are the most used and studied antiandrogens. Early development of flutamide (89) and its clinically proven efficacy led to the development of a series of its derivatives. Three non-

steroidal antiandrogens, i.e. flutamide (89), Anandron (114), and Casodex (108), have shown clinical benefits in the treatment of prostate cancer. The compound having the longest and largest clinical experience is flutamide, the first compound in prospective and randomized studies to be shown to prolong life in prostate cancer when associated with medical or surgical castration [46,110,111]. The clinical studies of the three non-steroidal antiandrogens will be

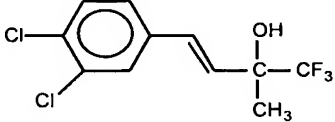
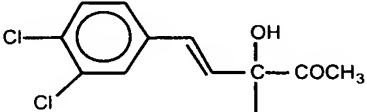
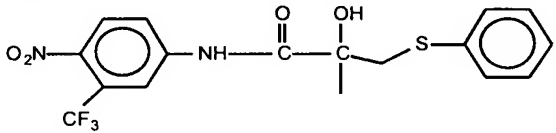
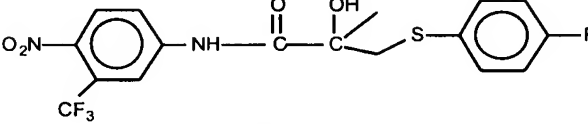
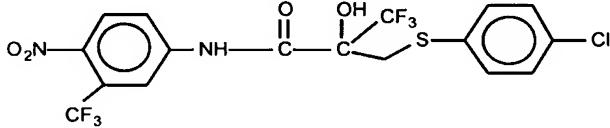
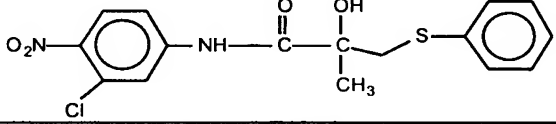
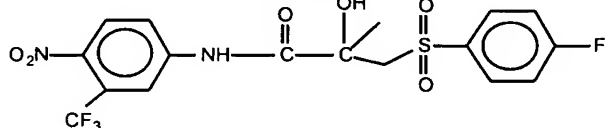
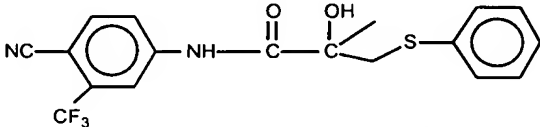
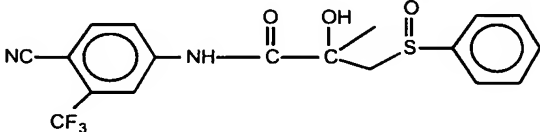
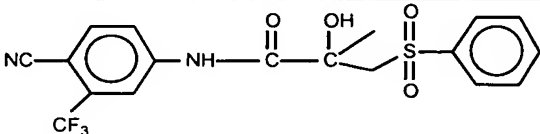
discussed in the later part of the review. Clinically, the most widely studied non-steroidal antiandrogen is flutamide, i.e. 2-methyl-4'-nitro-3'-(trifluoromethyl)propionanilide (**89**). 2-Hydroxylated derivative **90** of flutamide is the active metabolite. Structure-activity relationships infer that the most active compounds contain electron-withdrawing substituents in the aromatic ring and a branched alkyl chain α to the amide

carbonyl. Compounds with two electron-withdrawing substituents on the aromatic gave a better potency than the one substituent (compare **90:91**; RBA=2.1:0.2). When the CF₃ group was introduced at the C2 position of anilide having only one electron-withdrawing substituent, antiandrogenic activity of compound increased by five-fold (compare **91:92**; RBA=0.2:0.9).

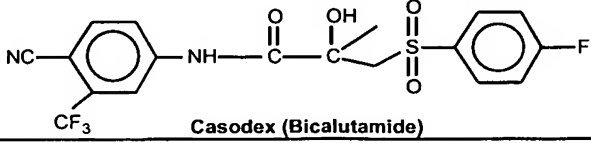
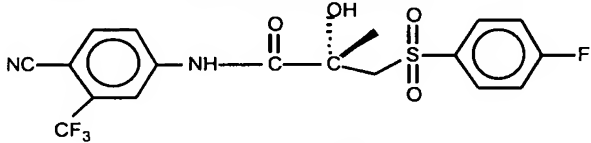
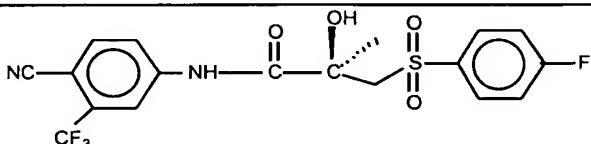
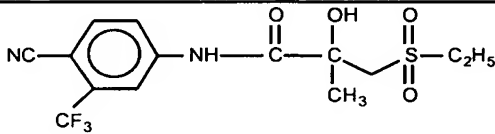
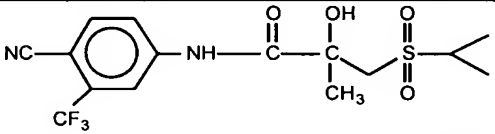
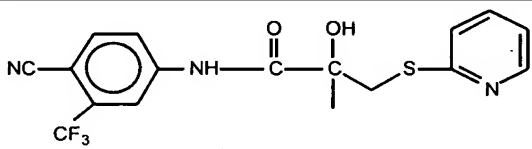
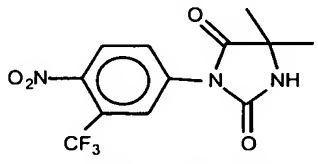
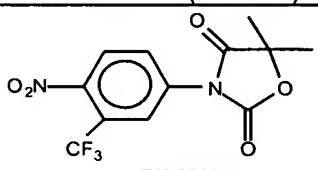
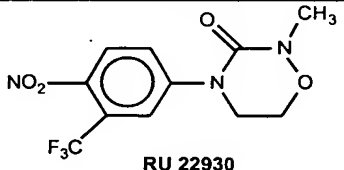
Table 8. Flutamide Derivatives as Antiandrogens

No	Structure	Antiandrogenic activity		Ref.
		RBA	ED ₅₀ (mg/kg/d)	
89	 Flutamide (Eulexin)	< 0.2	0.5, sc 80% @ 12, po	[112]
90	 Hydroxyflutamide	2.1 IC ₅₀ =72 nM (Shio. cell)	0.5, sc	[112]
91		< 0.2	-	[112]
92		0.9	5.0, sc	[112]
93		6.6	0.15, sc	[112]
94		15.1	0.12, sc	[112]
95		< 0.2	-	[112]
96	 AA560	-	93% @ 4, po	[113]
97	 RU 22273	<0.2	>10, sc	[112]

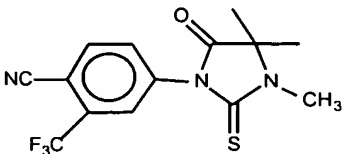
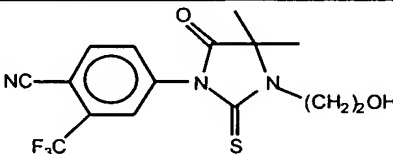
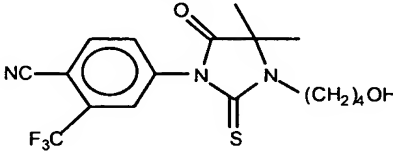
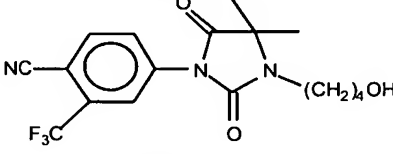
(Table 8). contd.....

No	Structure	Antiandrogenic activity		Ref.
		RBA	ED ₅₀ (mg/kg/d)	
98		<0.2	1.5, sc	[112]
99		0.4	0.2, po	[112]
100		-	1.0, po	[114]
101		-	1.1, po	[114]
102		-	23% @ 25(agonist)	[114]
103		-	0.5, po	[114]
104		-	0.4, po	[114]
105		-	1.7, po	[114]
106		-	1.4, po	[114]
107		-	1.8, po	[114]

(Table 8). contd.....

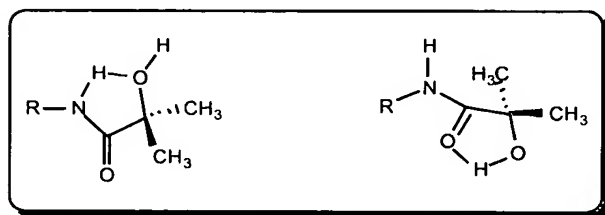
No	Structure	Antiandrogenic activity		Ref.
		RBA	ED ₅₀ (mg/kg/d)	
108	 Casodex (Bicalutamide)	IC ₅₀ =243 nM(Shio. cell)	0.5, po 64% @ 12, po	[114-116]
109		-	30.0, po	[116]
110		-	0.5, po	[116]
111		-	1.1, po	[114]
112		-	10, po	[114]
113		-	2.0, po	[114]
114	 RU 23908 (Anandron)	4.5(T: 100) IC ₅₀ =412 nM (Shio. cell)	58% @ 10, ip 94% @ 125, sc	[57,117-121]
115	 RU 22860	-	46% @ 10, ip	[57]
116	 RU 22930	-	≈60% red. of seb. g. @ 5 mg/ham.	[56]

(Table 8). contd.....

No	Structure	Antiandrogenic activity		Ref.
		RBA	ED ₅₀ (mg/kg/d)	
117	 RU 56187	⁹² (R1881: 290) (DHT: 180)	60 @ 1.0, po	[122]
118	 RU 57073	163	45 @ 1.0, po	[122]
119	 RU 59063	300	23 @ 1.0, po	[122]
120	 RU 58841	Ka=1.4 nM (T; Ka=0.7 nM) ham. (F.O.)	52% red. of F.O. @ 100 µg/ham.	[123]

In a compound **94**, where all three factors were present, the highest potency of the series was obtained. SAR revealed several factors which were responsible for high antiandrogenic activity.

1. An electron-deficient aromatic ring.
2. A powerful hydrogen bond donor group.
3. Fixed conformers involved in intramolecular hydrogen binding.



Replacement of the anilide by the alkene gave weakly active compounds such as **97**. The weak

activity of this compound can be attributed to the lack of possible intramolecular hydrogen binding or the poor hydrogen-bond donor capability. Thus, the only way to increase electron-donor ability is to introduce electron-withdrawing groups α to hydroxy. Thus, compounds **98** and **99** having powerful donor showed stronger *in vivo* activities.

In the Casodex series, compounds with the cyano or nitro group at the 4'-position and the chloro **103** or trifluoromethyl group **100** at the 3'-position of the anilide ring gave improved antiandrogenic activity. In general, 2-trifluoromethyl compounds, such as **102**, showed a mixed agonist/antagonist activity. Antiandrogenic activities of the sulfide **105**, sulfoxide **106** and sulfone **107** were comparable *in vivo* and sulfones were the major metabolites of the sulfides *in vivo*. In the case of the arylthio analogs, para-substituted groups decreased activity, except for chloro, which had little effect on potency. However, the fluoro group increased potency (compare compounds **107** and **108**). R-Casodex (**110**) was 60 times more

potent than S-Casodex (**109**) *in vivo*. In the alkylthio series, potency was optimum for the ethylthio analog **111** and decreased with increasing size of the alkyl group **112**.

Anandron [(5,5-dimethyl-3-{4'-nitro-3'-(trifluoromethyl)phenyl}-2,4-imidazolidine dione)] exhibited very low affinity for AR. While, it competes for either labelled T or RU 1881 binding to cytosol from castrated rat prostate. Anandron (**114**) has shown efficacy in the treatment of prostate cancer when added to castration [124]. Other modifications, such as nitrogen to oxygen **115**, has little effect on the potency of the compounds. Dichloro analog also showed similar efficacy. RBAs decreased on changing the alkyl group to an alcohol moiety. However, N-substituted arylthiohydantoin exhibited relatively high binding affinity to the rat androgen receptor. The RBA of RU 59063 (**119**) was 3 times that of testosterone, and 100 times that of non-steroidal antiandrogens. RU 59063 could be useful as a marker for AR. Furthermore, unlike other markers of AR, it was devoid of any binding to other steroid receptors. *In vivo*, another analog, RU 56187 (**117**) showed high antiandrogenic activity. In rats, compound **117** was 3 and 10 times more active than Casodex and Anandron, respectively. RU 58841 (**120**) displayed 2 times less affinity than T for the hamster flank organ (F.O.) androgen receptor. However, activity was similar for the human androgen receptor. *In vivo*, when applied topically, it provided 52% regression of flank organ area at 100 µg/hamster, while being devoid of antiandrogenic activity on other accessory sex organs.

Flutamide (**89**), Casodex (**108**), and Anandron (**114**) are the most studied pure antiandrogens *in vitro* and *in vivo* and are presently used in the treatment of prostate cancer. Recently, considerable efforts are being made for the development of pure antiandrogens more potent than flutamide, the first pure antiandrogen. Considering the rapidly rising interest in antiandrogens, especially following the demonstration that the addition of flutamide to an LHRH agonist prolongs life [51], comparison of the three was made, using the most appropriate *in vitro* and *in vivo* assays, to assess the biological characteristics of flutamide, Casodex, and Anandron. Thus, the two-fold stimulation of Shionogi cell proliferation caused by a 10-day exposure to 1 nM testosterone was competitively reversed by incubation with OH-FLU (**90**) ($IC_{50}=72$ nM), Casodex (243 nM) and Anandron (412 nM). Moreover, marked increase in GCDP-15 release induced by 1 nM testosterone was blocked by OH-FLU (35 nM), Casodex (142 nM) or Anandron (75 nM) in ZR-75-1 cells. These data demonstrate that the antiandrogenic activity of OH-FLU is 3.4-fold more potent than Casodex and 5.7-fold more potent than

Anandron. Furthermore, OH-FLU is 4.1 times more potent than that of Casodex in inhibiting testosterone-induced GCDP-15 secretion in ZR-75-1 cells. These data show a greater difference in potency in favor of OH-FLU [120]. The *in vivo* study also is in close agreement with the study *in vitro*. Flutamide is about three times more potent than Casodex in inhibiting ventral prostate and seminal vesicle weight in orchiectomized rats supplemented with $\Delta 4$ -dione [125].

Quinoline Derivatives as Antiandrogens (Table 9)

In 1998, workers from Ligand described a series of 1,2-dihydropyridono[5,6-g] quinoline derivatives as androgen receptor antagonists [126-132]. Quinoline **121** showed potent antiandrogenic activity along with antiprogesterin activity ($IC_{50}=49$ nM). Introduction of the C9 methyl group increased the selectivity between AR ($IC_{50}=23$ nM) and PR ($IC_{50}=3346$ nM) for compound **123**. Reduction of the 3,4-double bond of quinoline derivatives did not affect the potency. However, it greatly improved *in vivo* efficacy. In general, alkylation at the bottom part of molecules increased the activity. Methylation of the pyridone nitrogen **127-129** did not affect the antiandrogenic activity, but it enhanced the selectivity for AR over PR. A clear trend in activity did not arise from the alkylation of the quinoline nitrogen. However, methylation of both nitrogen **130** and **131** did not change the activity. Chemical substitution at C2 was essential for antiandrogenic activity. Substitution at the 2, 3, and 4 positions was tolerable.

Compounds **121**, **124**, **125**, and **127** were evaluated for their *in vivo* efficacy. Reduction of testosterone propionate (1 mg/kg, sc) induced ventral prostate in castrated rats at oral administration (30 mg/kg, po) of once a day for 3 days was 57, 55, 71, and 49%, respectively. Whereas flutamide gave a 100% reduction of VP weight, unsubstituted at the C4 position **125** gave the highest *in vivo* efficacy of the series.

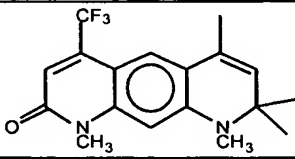
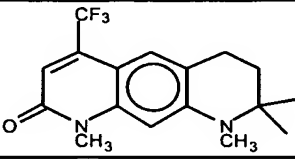
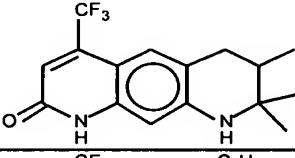
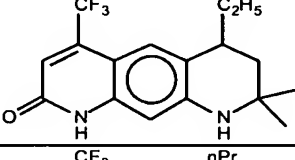
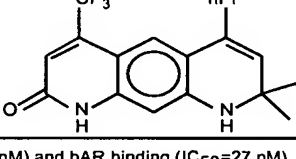
Cyclocymopol Analogs as Antiandrogens (Table 10)

Cyclocymopol monomethyl ether was isolated from a crude organic extract of the marine alga *C. barbata* and exhibited activity against the human androgen receptor and human progesterone receptor [133-136]. The acetate **139** of cyclocymopol monomethyl ether was a weak antiandrogen. However, the selectivity was significant for AR over PR ($IC_{50} > 10,000$ nM). SAR study revealed that bromo, hydroxy, and methoxy on

Table 9. Quinoline Derivatives as Antiandrogens

No	Structure	Antiandrogenic activity ^a		Ref.
		hAR in CV-1 cells (IC ₅₀ , nM)	hAR Binding (K _i , nM)	
121		28	115	[126]
122		26	76	[126]
123		23	82	[126]
124		22	85	[126]
125		27	26	[126]
126		35	9	[126]
127		34	81	[126]
128		73	46	[126]
129		31	40	[126]

(Table 9). contd.....

No	Structure	Antiandrogenic activity ^a		Ref.
		hAR in CV-1 cells (IC ₅₀ , nM)	hAR Binding (K _i , nM)	
130		46	39	[126]
131		19	17	[126]
132		30	73	[126]
133		27	54	[126]
134		159	650	[126]

^aOH-FLU: hAR (IC₅₀=15 nM) and hAR binding (IC₅₀=27 nM).

the aromatic ring were not necessary for the optimal activity (compare compounds **135** and **140**). In fact, replacement by the nitro group gave the most active compound **135** of the series. Moreover, the bromo group in the cyclohexyl ring was also not essential for activity (compare compounds **139** and **140**). Replacement of the exo-cyclic double bond **140** by cyclopropyl **142** or hydroxy **143** did not affect the activity. The gem-dimethyl group was essential for activity. Majority of the compounds also showed PR antagonist activity.

Phthalimide Derivatives as Antiandrogens (Table 11)

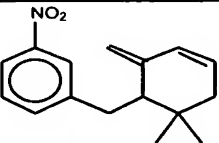
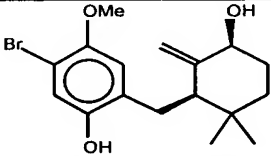
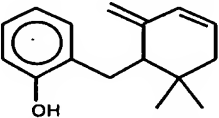
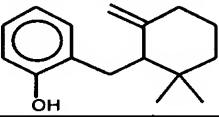
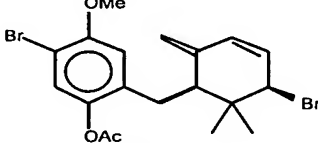
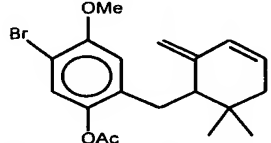
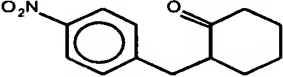
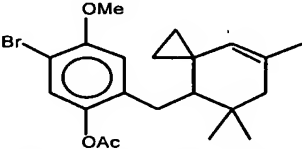
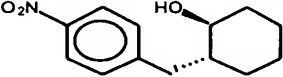
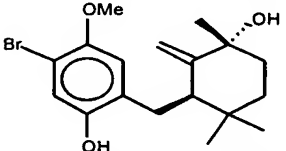
Antiandrogenic activity of various phthalimide analogs was evaluated against testosterone (10 nM)-stimulated Shionogi cells proliferation. Tetrafluorophthalimides **149-152** showed potent antagonistic activity. The activity of both enantiomers

was comparable. A chiral center next to the nitrogen atom was essential for the high potency of the fluoro compounds. Other phthalimide derivatives showed moderate activities and were comparable to flutamide (34% inhibition at 1 μ M). The inhibitory activity on androgen-induced activation of the nuclear androgen receptor was also evaluated in a CAT assay, and it well correlated to the antiandrogenic activity evaluated by the growth inhibition assay in Shionogi cells.

Other Non-steroidal Antiandrogens (Table 12)

1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethylene (**154**), the major and persistent metabolite of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) inhibits androgen binding to the androgen receptor. The *in vitro* potency of *p,p'*-DDE (**154**) was comparable to hydroxyflutamide (OH-Flu; 50% inhib. at 0.2 μ M). In adult rats, treatment with *p,p'*-DDE (200 mg/kg, po) for 4 days reduced seminal vesicle (16%) and ventral

Table 10. Cyclocymopol Analogs as Antiandrogens

No	Structure	Antiandrogenic activity	Ref
		hAR (IC ₅₀ , nM) ^a	
135		66	[135,136]
136		180	[135,136]
137		210	[135,136]
138		220	[135,136]
139		230	[135,136]
140		240	[135,136]
141		250	[135,136]
142		250	[135,136]
143		290	[135,136]
144		300	[135,136]

^aAntiandrogenic activity was performed on the human androgen receptor utilizing CV-1 cells.

Table 11. Phthalimide Derivatives as Antiandrogens

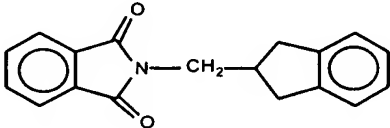
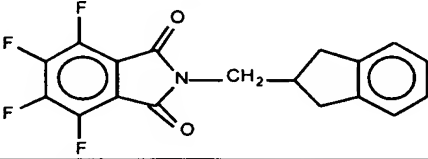
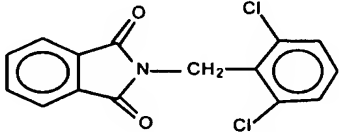
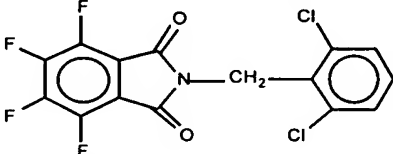
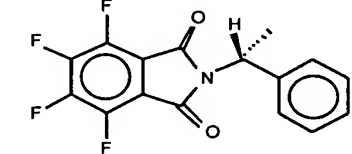
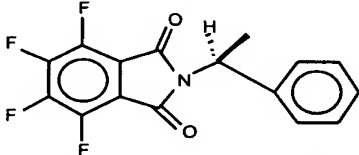
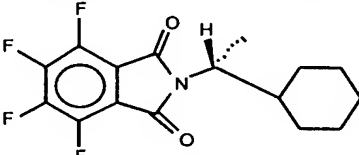
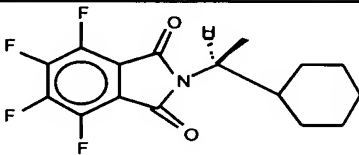
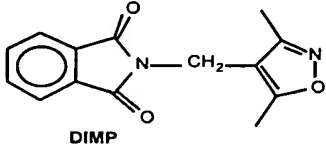
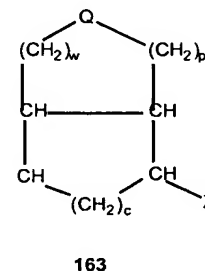
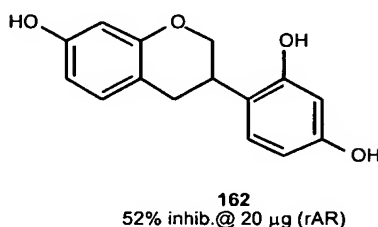
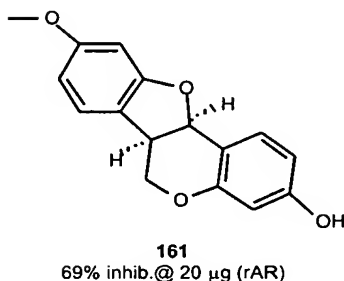
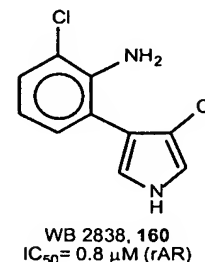
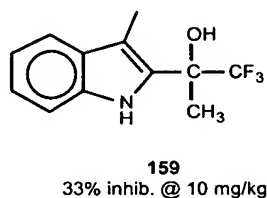
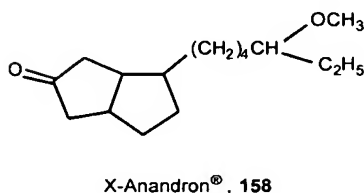
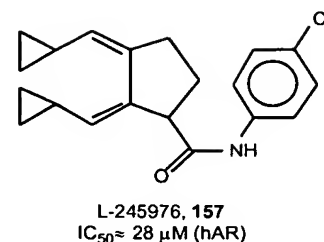
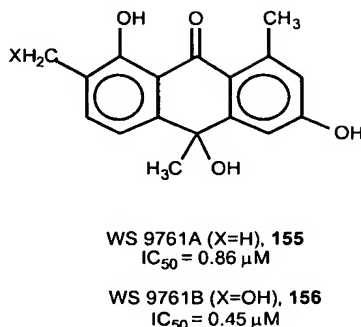
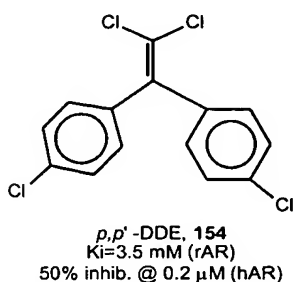
No	Structure	Antiandrogenic activity	Ref.
		Shio cells (% inhib. at 1 μ M)	
145		44	[137]
146		39	[137]
147		31	[137]
148		55	[137]
149		84	[137]
150		97	[137]
151		98	[137]
152		95	[137]
153	 DIMP	(RBA: 2.55) (R1881: RBA:100)	[109]

Table 12. Other Non-steroidal Antiandrogens

prostate weight (30%). These results support the hypothesis that the antiandrogenic effects of DDT on the male reproductive system are mediated by *p,p'*-DDE [138]. Compounds **155** and **156**, isolated from the fermentation broth of a *Streptomyces* strain microorganism, were moderately active as antiandrogens [139,140]. In DDT1 cells, L-245976 (**157**) completely blocked the action of testosterone (10 nM) at 10 μM , whereas hydroxyflutamide at 1 μM had a similar effect. Moreover, compound **157** also exhibited low affinity for AR ($\text{IC}_{50} \approx 28 \mu\text{M}$) compared to hydroxyflutamide ($\text{IC}_{50} \approx 100 \text{ nM}$) [141]. Antiandrogen **158** blocked DHT binding to androgen receptors from fibroblasts of frontal skin from alopecia patients by 80%, and of fibroblasts from facial skin by 78-93% [142]. Other non-steroidal antiandrogens **159-163** have also been reported to display good antiandrogenic activity [112,143-145].

Clinical Results

Over the past thirty years, little progress has been made in the development of potent antiandrogens.

Few of them have shown a promising response in pre-clinical studies. Moreover, clinical studies remain to be done to further define their efficacy in the treatment of prostate cancer and skin related diseases. So far, flutamide and its derivatives have displayed good clinical benefits in human, and are extensively used. The clinical results of these antiandrogens are summarized in this last section of the review.

Cyproterone Acetate (CPA; 29; Table 3)

An EORTC study compared the clinical results of CPA, DES and MPA in 210 patients. The efficacy of CPA and DES was similar when compared for progression and survival rates while MPA was less effective [146,147]. When compared to flutamide, this progestin has significant intrinsic androgenic and estrogenic activities. CPA causes estrogen-like complications such as thrombosis, cardiovascular side effects, gynecomastia, and adverse effects on serum lipoproteins [148-150]. Virilization effects were seen in all the female fetuses examined when pregnant guinea pigs were given cyproterone acetate, thus providing

early evidence of androgenic activity [151]. The effects of flutamide and the steroidal derivatives, cyproterone acetate, chlormadinone acetate, megestrol acetate and medroxyprogesterone acetate were compared *in vivo* in female nude mice bearing androgen-sensitive Shionogi tumors. All steroidal compounds stimulated tumor growth while flutamide had no stimulatory effect [51]. Thus, CPA due to its intrinsic properties stimulates androgen-sensitive parameters and cancer growth. Cyproterone acetate added to castration has never been shown in any controlled study to prolong disease-free survival or overall survival in prostate cancer when compared with castration alone [152-155].

Flutamide (89; Table 8)

The first pure antiandrogen was discovered in 1967 by Neri et al. at Schering-Plough. Flutamide, an orally active antiandrogen, is rapidly metabolized to the active compound hydroxyflutamide, which accounts for almost all metabolites of flutamide present in the circulation. For the treatment of cancer, flutamide is administered at the dose of 250 mg every 8 hours. For the treatment of hirsutism and androgenic alopecia in women, a twice daily dose of 250 mg was used [7]. Two large-scale double blind studies have shown that combination of flutamide and medical castration increases the number of responders, and most importantly increases overall survival by an average of 7.3 months when compared with an LHRH agonist and orchiectomy, respectively [110,111]. These studies demonstrate that pure antiandrogens should always be given in combination with medical (LHRH agonist) or surgical (orchiectomy) castration as first treatment at the start of therapy.

Since localized disease provides the only opportunity for cure of prostate cancer, the combination therapy was next administered to patients at earlier stages of the disease. Randomized studies performed in patients have recently demonstrated that combination therapy administered for 3 months before radical prostatectomy increases the proportion of patients having organ-confined disease by about 50% while the same approach associated with radiotherapy has been shown to delay the time to progression [51,156]. Loose bowel movements or diarrhea is observed in 5-9% of cases. No cardiovascular effects are observed. When given in combination, no gynecomastia or breast tenderness is observed.

Anandron (114; Table 8)

Anandron (Nitulamide) is well absorbed after oral administration. Anandron is usually administered at a daily dose of 300 mg per day for one month followed by

the daily maintenance dose of 150 mg. Nitulamide given in association with orchiectomy in advanced prostate cancer has shown, in randomized and prospective studies, a greater proportion of responders, a longer duration of disease-free survival and an increase of an average of 5.4 months and 7.3 months in overall survival compared to orchiectomy alone [157,158]. Other data have shown an improved response and an improved quality of life [159,160]. In analogy with flutamide, the benefits of Anandron are much superior when given as first treatment. Visual adaptation to darkness is impaired in 20-40% of patients. Mild gastrointestinal disturbances were found in a few patients and interstitial lung disease is unfrequent.

Casodex (108; Table 8)

Casodex (bicalutamide) is an orally bioavailable and well absorbed antiandrogen. In a randomized, multicenter and open study in 376 patients with metastatic prostate cancer, the effect of Casodex (50 mg/day) was compared with orchiectomy. At 3 months, PSA was reduced by 86% in the Casodex group and by 96% in the orchiectomy group [161].

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Oral Contraceptives in the Treatment of Acne

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ABSTRACT

Oral contraceptives (OCs) can reduce acne by lowering the production of adrenal and ovarian androgens, by inhibiting 5-alpha-reductase, which in turn, reduces the levels of dihydrotestosterone, and by stimulating sex hormone binding globulin (SHBG), thus reducing the levels of free testosterone. In newer OCs, such as Tricyclen and Diane-35, the progestin component is minimally androgenic and anti-androgenic respectively, thereby enhancing the favorable profile of these products in the treatment of hyperandrogenic disorders, including acne. The efficacy of these agents and their long-term safety profile supports their use in various grades of acne in females:

- as adjunctive therapy to topical agents for women with mild non-scarring acne desiring oral contraception
- as primary therapy for patients with moderate non-scarring acne in combination with topical therapy and systemic antibiotics
- as one of two preferred methods of contraception in patients with scarring and severe inflammatory acne being treated with systemic isotretinoin.

KEY WORDS: acne, oral contraceptives, progestin

Acne is initiated by the effect of androgens on pilosebaceous units resulting in sebaceous hypersecretion and follicular occlusion. Hormonal therapy in acne is, therefore, rationally directed at interrupting this initial phase in the sequence of acne pathogenesis.

Oral contraceptives have been available since 1960, and have evolved to contain less estrogen, thus minimizing the risk of thromboembolic events, hepatic tumors, hypertension, and altered glucose metabolism. As well, present day OCs include progestins, which have less intrinsic androgenicity. These developments in OC pharmacology also led to their usefulness in treatment across the spectrum of acne severity in females.¹

Hormonal Pathways in Acne Pathogenesis

Androgens that are relevant in acne pathogenesis include dihydrotestosterone (DHT), testosterone (T), androstenedione (A) and dehydroepiandrosterone-sulfate (DHEA-S). The production of these androgens from ovaries and adrenal glands is mediated by

gonadotrophins. Testosterone is converted to the biologically more potent 5-dihydrotestosterone (5-DHT) by 5-alpha-reductase. The bioavailable testosterone fraction is considered to be biologically active and comprises the free fraction of testosterone and the fraction bound by albumin. Levels of free testosterone are inversely related to levels of sex hormone binding globulin (SHBG).

Oral Contraceptives

OCs, which contain estrogen and progestins, directly affect androgen physiology and can therefore impact acne. Potential mechanisms of the therapeutic effect of estrogens include:

- decreased production of adrenal (DHEA-S) and ovarian androgens (A, T)
- inhibition of 5-alpha-reductase leading to the reduction of DHT levels
- stimulation of SHBG, reducing levels of free T.

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19-nortestosterone derivatives		Progesterone derivatives
Gonanes (norgestrel related)	Estranes (norethindrone related)	
Norgestrel	Norethindrone	Cyproterone acetate
Levonorgestrel	Norethindrone acetate	
Desogestrel	Ethinodiol diacetate	
Gestodene	Lynestrenol	
Norgestimate		

Table 1: Overview of Progestins

Progestins vary in their androgenic potential and may therefore have variable effects on acne. The most commonly used progestins in OCs are 19-nortestosterone derivatives (see Table 1).² Progestins with the lowest androgenic potential (e.g., desogestrel, norgestimate, cyproterone acetate) are more appropriate in the treatment of acne and other hyperandrogenic disorders.

Hormonal Preparations Approved for Treatment of Acne

1. Ethinyl estradiol 0.035mg with norgestimate in increasing doses of 0.180mg/0.215mg/0.250mg (*Tricyclen*):

Norgestimate has low intrinsic androgenicity with low binding affinity for androgen receptors, whereas it is strongly selective

and avidly bound to progesterone receptor sites. Two 6-month, randomized, double-blind placebo-controlled trials involving 507 females with moderate acne showed clinically and statistically significant reduction of inflammatory lesions and total lesion counts.^{3,4} Moderate acne was defined as 6-100 comedones, 10-50 papules or pustules, and no more than 5 nodules. The mean decrease in inflammatory lesion count was 12, or 62% from baseline (compared to 8 lesions or 39% for placebo), and the decrease in total lesion count was 29, or 53% (compared to 14, or 27% in placebo). No significant changes for nodules were noted. A 50% reduction in total lesion count was attained between the 4th to 6th month of treatment, with a plateau of effect attained at 6 months.

	Erkkola et. al (1990) ¹²	Aydinlik et. al (1990) ¹³	Gollnick et. al. (1998) ¹⁴
Randomized?	Yes	No	No
Placebo-controlled?	No Compared to desogestrel/ethinyl estradiol*	No	No
Blinded?	No	No	No
Standard Dermatological Assessment and Outcome Endpoints	No "healing/improvement" "complete healing"	No "healing/improvement" "definitive healing" "complete healing"	Yes: Lesion counts and acne grading
Sample Size At Start/At Completion	162/133	1161/1071 (after 6 months); 850 (after 12 months); 192 (after 36 months)	890/794
Duration of Treatment	9 months	Maximum 36 months	6 months
Efficacy	Complete healing: 60% Healing/Improvement: 81%	Healing/improvement: 72% (at 6 months); 90% (at 12 months)	Lesion count reduction 73%; reduction in acne grade in 64%
Adverse Events		Headache (7%), nausea (5%), breast tension (13%), melasma (7%)	Breast tenderness (12%), headache (9%), nausea (6%), nervousness (4%), dizziness(3%)

Table 2: Summary of Pivotal Studies for *Diane-35*

* desogestrel 0.15 mg with ethinyl estradiol 0.03 mg

2. Ethinyl estradiol 0.035mg and cyproterone acetate 2mg (Diane-35):

Cyproterone acetate (CPA) is an analogue of hydroxyprogesterone and has progestational activity. It acts as a potent antiandrogen by competitive inhibition of T and DHT binding to the androgen receptors, and by inhibiting gonadotropin secretion. It is currently available in a dose of 2mg CPA in combination with ethinyl estradiol (Diane-35). Higher doses of CPA (50-100mg/d) may be required in treatment of more refractory acne or if associated with hyperandrogenization.⁵

The effectiveness of Diane-50 (containing 0.05mg of ethinyl estradiol and 2mg of CPA) for the treatment of acne has been demonstrated in placebo-controlled and antibiotic-comparative trials. Two randomized controlled trials compared Diane-50 to systemic antibiotics in the treatment of acne. In a 6-month trial of 78 patients randomized to minocycline 50mg po bid or Diane-35, papules were reduced by 73% and 70%, and pustules by 77% and 83%, respectively.⁶ A similar 6-month trial evaluating Diane-50 compared to tetracycline 500mg po bid in 92 women showed reduction in lesion counts of 74% and 68% respectively.⁷

Two randomized controlled trials compared Diane-35 to Diane-50, one for 9 months and the other for 12 months. They demonstrated that these preparations were similarly effective in the treatment of acne.^{8,9} In a 6-month study of 133 patients randomized to treatment with Diane-35, Diane-50 or levonorgestrel 0.15mg/ethinyl estradiol 30mg, the reduction in acne lesions from baseline was 72%, 70%, and 35% respectively.¹⁰

The three pivotal studies referred to in the Canadian product monograph¹¹ for Diane-35 are summarized in Table 2.¹²⁻¹⁴ One trial was randomized, comparing Diane-35 to desogestrel 0.15 mg/ethinyl estradiol 0.03mg, but was unblinded, introducing the potential for observer or patient bias. Standard dermatological outcome parameters for acne, such as lesional counts or change in acne grade, were used in only one study. In that study fifty percent reduction in lesion counts was attained between the 2nd to 4th month of treatment.¹⁴ Two studies used non-standard, subjective outcome parameters such as "healing/improvement", "complete healing" and "definitive healing" which are of limited utility in outcome assessment across studies.

Summary

Currently available OCs such as Tricyclen and Diane-35, containing progestins with minimal androgenic and anti-androgenic potential respectively, provide an important therapeutic option for women with acne. Their proven efficacy and long-term safety profile support their use for various grades of acne in females.

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Drug Treatments For Skin Disease Introduced in 2000

Drug Class	Generic/Trade/ Company Names	Labelled Indication	Approving Regulatory Agency
Anti-acne Agent	Chloramphenicol, Sulphur, Hydrocortisone Acetate, 2-butoxyethyl Nicotinate, Allantoin <i>Actinac</i> Aventis Pharma	• For the treatment of mild to moderate acne	TPP - Canada
	Clindamycin Phosphate & Benzoyl Peroxide <i>Clindoxyl Gel</i> Stiefel Canada	• For the treatment of mild to moderate acne	TPP - Canada
	Clindamycin Phosphate <i>Clindasol Cream 1% & Clindets Pledgets</i> Stiefel Canada	• For the treatment of mild to moderate acne	TPP - Canada
Antibacterial Agent	Levofloxacin Tablets/Injection <i>Levaquin Tablets/Injection</i> Ortho Pharmaceuticals	• Additional indication for the treatment of complicated skin and skin structure infections at a higher dose of 750mg once daily	US FDA
	Linezolid <i>Zyvox</i> Pharmacia & Upjohn	• For the treatment of complicated skin/skin structure infections, infections caused by vancomycin resis- tant <i>Enterococcus</i> , and hospital acquired pneumonia	US FDA
	Silver Sulfadiazine <i>Flamazine</i> Smith & Nephew	• Previously available for superficial skin infections	TPP - Canada
Antifungal Agent	Amphotericin B Liposome for Injection <i>Ambisome</i> Fujisawa Canada	• For the treatment of systemic or disseminated infec- tions due to <i>Candida</i> , <i>Aspergillus</i> or <i>Cryptococcus</i> in patients refractory to, or intolerant of conventional amphotericin B therapy, or suffer renal impairment.	TPP - Canada
	Ciclopirox Olamine 1% <i>Loprox Cream, Loprox Lotion</i> Dermik Laboratories	• For the treatment of fungal infections	TPP - Canada
	Ketoconazole 2% Cream Teva Pharmaceuticals	• For the treatment of fungal infections	US FDA
	Terbinafine HCl 1% Solution <i>Lamisil</i> Novartis	• For the treatment of interdigital athlete's foot, jock itch and ringworm. Switched from prescription to OTC status	US FDA
	Terbinafine HCl <i>Gen-Terbinafine 250mg Cap</i> Genpharm	• For the treatment of interdigital athlete's foot, jock itch and ringworm. Switched from prescription to OTC status	TPP - Canada
	Terbinafine HCl <i>Novo-Terbinafine 125- 250mg Cap</i> Novopharm	• For the treatment of interdigital athlete's foot, jock itch and ringworm. Switched from prescription to OTC status	TPP - Canada
	Terbinafine HCl <i>Terbinafine - 250</i> Pro Doc Limitée	• For the treatment of fungal infections	TPP - Canada
Antihistamines	Loratadine <i>Claritin</i> Schering-Plough	• For the treatment of chronic idiopathic urticaria and seasonal allergies in children ≥ 2 years of age	US FDA
	Trimeprazine Tartrate <i>Panectyl</i> Aventis Pharma	• Antipruritic	TPP - Canada
Anti- inflammatory Agent	Amlexanox 5% Paste <i>Aphera</i> Access Pharmaceuticals	• For the treatment of aphthous ulcers (canker sores)	TPP - Canada
Antipsoriatic Agent	Calcipotriol <i>Dovonex Cream & Ointment</i> Leo Pharma	• New indication for the treatment of psoriasis, used in combination with topical corticosteroids, cyclosporin A, acitretin, and phototherapy (PUVA or UVB)	TPP - Canada

Drug Class	Generic/Trade/ Company Names	Labelled Indication	Approving Regulatory Agency
Antirejection Agent	Cyclosporin <i>Gengraf</i> Abbott Laboratories	• For the prevention of organ rejections in kidney, liver and heart transplants. It is the bioequivalent to <i>Neoral</i> (Novartis Pharmaceuticals)	US FDA
Antirosacea Agent	Metronidazole <i>Rosazol Topical Cream, 1%</i> Stiefel	• For the treatment of rosacea	TPP – Canada
Antiviral Agent	Docosanol Cream 10% <i>Abreva</i> Glaxo Smith Kline	• For the treatment of recurrent oral facial herpes simplex infections	US FDA (OTC)
	Interferon-Alpha <i>Veldona</i> Amarillo Bioscience	• For the treatment of papillomavirus warts in the oral cavity of HIV positive patients	US FDA (Orphan Drug Designation)
Corticosteroids	Flumethasone Pivalate <i>PMS-FLUMETHASONE-CLIOQUINOL</i> Pharmascience	• For the treatment of otitis externa, and otomycosis due to <i>aspergillus niger</i> . It is the generic form of <i>Locacorten Vioform</i> (Novartis)	TPP – Canada
	Prednicarbate 0.1% <i>Dermatop Emollient Cream & Dermatop Ointment</i> Dermik Laboratories	• For the relief of the inflammatory and pruritic manifestations of acute and chronic corticosteroid-responsive dermatoses	TPP – Canada
Dermatosclerosis Agent	Halofuginone Collgard Biopharmaceuticals	• For the treatment of scleroderma	US FDA (Orphan Drug Designation)
HIV/AIDS	Amprenavir <i>Agenerase</i> Glaxo Wellcome	• For use in combination with other anti-retroviral agents for the treatment of PI-experienced HIV infected adults and children ≥ 4 years of age	CPMP – Europe
	Lamivudine, Zidovudine & Abacavir <i>Trizivir</i> Glaxo Wellcome	• For the treatment of HIV infection	US FDA
Hormonal Preparations	Testosterone Gel <i>AndroGel 1%</i> Unimed Pharmaceuticals	• For the treatment of low testosterone levels linked with reduced sex drive/impotence, reduced lean body mass, reduced bone density, and lowered mood/energy levels in men who have not had breast or prostate cancer.	US FDA
Immunomodulators	Tacrolimus Ointment <i>Protopic</i> Fujisawa	• For the treatment of moderate-to-severe atopic dermatitis or eczema	US FDA
Keratolytic Agent	Diclofenac Sodium <i>Solaraze (US)</i> <i>Solaraze (Canada)</i> SkyePharma PLC	• For the treatment of actinic keratosis	US FDA TPP – Canada
	5-fluorouracil <i>Microsponge</i> <i>Solex</i> Advanced Polymer Systems / Dermik Laboratories	• For the treatment of actinic keratoses	US FDA
Neurotoxin	Botulinum Toxin Type B <i>Myobloc</i> Elan Corporation	• For the treatment of patients with cervical dystonia to reduce the severity of associated abnormal head position and neck pain. Past experience suggests that it will likely attract off-label use for wrinkle correction.	US FDA
Oncologic Agent	Alitretinoin 0.1% Gel <i>Panretin</i> Ligand Pharmaceuticals	• For the treatment of cutaneous lesions from AIDS-related Kaposi's sarcoma.	CPMP – Europe
	Bexarotene Capsules <i>Targretin</i> Ligand Pharmaceuticals	• For the treatment of all stages of cutaneous T-cell lymphoma in patients who are refractory to > 1 prior systemic therapy.	US FDA
	Bexarotene 1% Gel <i>Targretin</i> Ligand Pharmaceuticals	• For the treatment of cutaneous lesions in patients with early-stage cutaneous T-cell lymphoma who cannot tolerate other therapies.	US FDA

Drug Class	Generic/Trade/ Company Names	Indication	Approving Regulatory Agency
<i>Oncologic Agent</i>	Bleomycin Gensia Sicor Pharmaceuticals	• This generic injection form of Bristol-Myers Squibb's bleomycin (<i>Blenoxane</i>) tentatively approved for the management of squamous cell carcinoma, lymphomas and testicular carcinoma	US FDA
	Cyclophosphamide 25mg and 50mg Tablets Roxane Laboratories	• For treatment of certain forms of cancer to be used in combination with other antineoplastic therapies	US FDA
	Hydroxyurea Barr Laboratories	• This generic formulation approved for use in the treatment of melanoma	US FDA
	Paclitaxel <i>Paxene</i> IVAX/Bristol-Myers Squibb	• For the treatment of AIDS-related Kaposi's sarcoma in patients who have failed prior liposomal anthracycline therapy.	TPP - Canada
<i>Pediculocides</i>	Piperonyl Butoxide/Pyrethrins <i>Rid Mousse</i> Soltec Research	• For the treatment of head, pubic (crab), and body lice	US FDA
<i>Photo-aging</i>	Tretinoin Cream <i>Renova 0.02%</i> Ortho Pharmaceuticals	• For reducing fine facial wrinkles associated with chronic sun exposure and the natural aging process.	US FDA
	Tretinoin Cream <i>Renova 0.05%</i> Ortho Pharmaceuticals	• For use in the mitigation of fine wrinkles, mottled hyperpigmentation and tactile roughness of facial skin	US FDA
<i>Scalp Dermatoses</i>	Clobetasol Propionate <i>Olux Foam 0.05%</i> Connetics	• For the short-term topical treatment of inflammatory and pruritic manifestations of moderate-to-severe corticosteroid-responsive dermatoses of the scalp.	US FDA
<i>Topical Anesthetic</i>	Lidocaine & Procaine <i>EMLA Cream & Patch</i> AstraZeneca	• Topical anesthetic for dermal analgesia	TPP - Canada
<i>Transdermal Agent</i>	Estradiol Transdermal System <i>Vivelle</i> Novogyne Pharmaceuticals	• For the additional indication of postmenopausal osteoporosis for this transdermal estrogen patch	US FDA
<i>Urticaria</i>	Fexofenadine HCl <i>Allegra</i> Aventis Pharmaceuticals	• For the additional treatment of uncomplicated skin manifestations of chronic idiopathic urticaria in adults and children ≥ 6 years of age	US FDA
<i>Vaginal Preparations</i>	Butoconazole Nitrate <i>Gynazole 2% Vaginal Cream</i> KV Pharmaceuticals	• For the treatment of vaginal yeast infections using only one dose.	US FDA
	Clotrimazole 2% <i>Clotrimazole 2% Three Day Vaginal Cream</i> Taro Pharmaceuticals	• OTC for the treatment of vaginal yeast infections	US FDA
	Estradiol Vaginal Ring <i>Estring</i> Pharmacia and Upjohn	• For the treatment of urogenital symptoms associated with post-menopausal vaginal atrophy	US FDA
<i>Wound Care</i>	Tissue Engineered Collagen Matrix <i>Oasis Wound Dressing</i> COOK's	• For the treatment of full-thickness skin injuries	US FDA
	Graftskin <i>Apligraf</i> Novartis Pharmaceuticals	• For expanded use with conventional diabetic foot ulcer care in the treatment of diabetic foot ulcers > 3 weeks in duration	US FDA
	"Intelligent" Dressing <i>Acemannan Hydrogel</i> Carrington Laboratories	• For the management of postsurgical incisions, first- and second-degree burns, arterial and venous stasis ulcers, pressure ulcers, and foot ulcers	US FDA

TPP: Health Canada, Ottawa - Therapeutic Products Program

FDA: United States Food and Drug Administration

CPMP: European Union's Committee for Proprietary Medicinal Products

Roche Introduces New Drug Warnings for Accutane in US

As a result of recent meetings held by the US FDA, Roche has introduced further steps to inform doctors and patients about possible side effects by their anti-acne drug *Accutane* (isotretinoin), such as birth defects, mental disorders and suicide.

Doctors must now make sure their patients sign a consent form that explains the drug's possible risks before they can write the prescription for this drug. As well, each prescription for *Accutane* will include a consumer-friendly pamphlet that details the benefits and the possible side effects.

Accutane labelling already includes both birth defects and mental disorders. Roche has taken several steps to publicize the drug's potential teratogenic effects, including an elaborate pregnancy prevention program, as well as a tracking program monitored by the Slone Epidemiology Unit in Boston, Massachusetts. However, in spite of this, some women who were taking *Accutane* became pregnant each year.

In addition to addressing birth defects as a possible side effect, the new consent forms also advise patients to look for signs of depression, e.g., feelings of sadness, irritability, fatigue or loss of appetite. Health officials are not certain whether *Accutane* does indeed cause problems with depression, suicidal thoughts and mood disorders.

American Academy of Dermatology Issues Policy Statement on Accutane

In November 2000, the American Academy of Dermatology (AAD) issued a new policy statement on *Accutane* (isotretinoin, Roche), which reads as follows:

- The Academy is committed to the safe and responsible use of isotretinoin.
- The Academy calls on the FDA not to limit the ability of qualified patients, in consultation with their physician, to receive isotretinoin.
- The Academy supports more education for physicians and patients on potential pregnancy hazards.
- The Academy opposes the inclusion of isotretinoin on a list of drugs that can only be obtained by registered physicians and patients.
- The Academy calls for a study designed to determine if there is a direct causative link between isotretinoin and psychiatric events.
- The Academy calls upon the American Medical Association to support its efforts to ensure the continued accessibility to, and medically appropriate use of, isotretinoin.

The AAD indicated that patient safety is its primary concern, and feels that education of physicians and their patients, rather than regulation, is the best way to ensure safe and effective results with this medication.

Update on Drugs

Class	Name/Company	Approval Dates and Comments
Antihistamine	Loratidine <i>Claritin</i> Schering-Plough	Schering-Plough submitted an abridged application to the US FDA and to the European Regulatory Committee (CPMP) in January 2001, to market their non-sedating antihistamine in a rapidly disintegrating tablet form for the treatment of seasonal allergic rhinitis and chronic idiopathic urticaria in adults and children ≥ 12 years of age.
Antiviral Agent	Varicella Zoster Immune Globulin <i>VariZIG</i> Cangene	TPP-Canada approved this hyperimmune product in January 2001. <i>VariZIG</i> is a highly purified and specialized antibody against the varicella zoster virus that causes chicken pox.
Antihistamine	Levocetirizine <i>Xyzal/Xusal</i> Sepracor	The German Health Authorities approved this new generation antihistamine in January 2001, for the treatment of seasonal allergic rhinitis, perennial allergic rhinitis and chronic idiopathic urticaria.
Enzyme Replacement Therapy	Agalsidase Beta <i>Fabrazyme</i> Genzyme General	The US FDA completed its review of the biologics licensing application in December 2000, for this investigational enzyme replacement therapy for Fabry disease. Genzyme must supply additional data and conduct a Phase IV trial before the US FDA will give their approval.
Atopic Dermatitis Agent	ASM 981 Cream 1% Novartis Pharmaceuticals	An NDA was submitted to the US FDA in December 2000, for this non-steroid, skin-specific cytokine inhibitor for the treatment of atopic dermatitis or eczema.

Drug News

Antiviral Agent	Docosanol 10% Cream (<i>Abreva</i>) was launched in January 2001, by SmithKline Beecham. It is the first OTC cold sore medication to be approved by the US FDA for reducing the healing time and duration of symptoms.
Antiviral Agent	Novartis Pharmaceuticals has acquired the antiviral products <i>Famvir</i> (famciclovir) and <i>Denavir</i> (penciclovir) from SmithKline Beecham for the treatment of herpes.
Atopic Dermatitis Agent	According to results presented at the annual meeting of the American College of Asthma, Allergy and Immunology in October 2000, topically applied strontium salts may be effective for treating itch and sensory irritation. Dr. G.S. Hahn from Cosmederm Technologies stated that when atopic subjects were treated with 20% strontium nitrate in a 5% ethanol/water vehicle vs. the vehicle alone, 70% of strontium treated sites had less itch than the vehicle treated sites. Topically applied strontium is very safe and can be found in more than 30 cosmetic and dermatological products sold around the world.
Allergic Contact Dermatitis	French researchers report that skin painting, also known as pseudo-tattooing, can lead to allergic contact dermatitis. Skin painting is done using henna, a hair dye preparation, and C.J. Le Coz, et al* stated that when henna is used on the skin, it can cause sensitization to chemical coloring agents, such as p-phenylenediamine and diaminobenzenes or diaminotoluenes. *Arch Dermatol 136(12):1515-7 (2000 Dec)

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5 α -Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats

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Tobias, J. H., A. Gallagher, and T. J. Chambers. 5 α -Dihydrotestosterone partially restores cancellous bone volume in osteopenic ovariectomized rats. *Am. J. Physiol.* 267 (Endocrinol. Metab. 30): E853–E859, 1994.—Although androgens are thought to be important for skeletal maintenance in females and males, little is known about the mechanisms involved. To investigate this question further, we examined the effects of administering 0.01, 0.1, or 1.0 mg/kg 5 α -dihydrotestosterone (DHT) for 60 days on the skeleton of ovariectomized rats. Treatment was delayed until 90 days after ovariectomy to enable bone loss to stabilize. We found that ovariectomy markedly reduced cancellous bone volume of the proximal tibial metaphysis due to a combination of loss and thinning of trabeculae. Cancellous bone volume was partially restored by all doses of DHT, with trabecular thickness, but not number, returning to that of sham-operated animals. DHT also stimulated longitudinal bone growth and endosteal and periosteal bone formation and suppressed histomorphometric indexes of cancellous bone resorption. This suggests that DHT influences skeletal metabolism in osteopenic ovariectomized rats both by stimulating bone formation and suppressing resorption, although it is unclear which, if any, of these actions predominate at cancellous sites.

androgens; bone formation; bone resorption

HYPOGONADISM IN MEN is associated with osteopenia (9, 13) that is partially reversed by testosterone treatment (8, 13), suggesting that androgens play a physiological role in skeletal maintenance in the male. Furthermore, this role may extend to females, since serum testosterone has been found to correlate with bone mass in premenopausal women (3). In addition, investigations into the use of androgen-related anabolic steroids in the treatment of women with postmenopausal osteoporosis generally report an increase in bone mass (6, 11, 17). However, studies in both men (1, 10, 24) and women (2, 5, 6) have failed to resolve whether this reflects a predominantly anabolic or antiresorptive action.

The mechanisms by which androgens influence skeletal metabolism have been addressed using male rats, in which androgens are also important for skeletal maintenance (25, 27–29). As in clinical studies, androgens were found both to stimulate bone formation (30) and to suppress resorption (27, 28). Moreover, in a recent study, administration of the androgenic antagonist flutamide was found to cause loss of skeletal calcium from female rats, attributed to a reduction in bone formation (12). However, although this suggests that androgens are also important in regulating bone formation in female rats, no study has examined the effect of androgens on specific skeletal sites in these animals.

To investigate the mechanisms involved in androgen's action on the female skeleton, we therefore carried out a

histomorphometric assessment of the effects of androgen administration in female rats. Because the ovaries are the major source of androgens in ovulating animals, we used ovariectomized (OVX) rats, delaying treatment until 90 days after ovariectomy to enable cancellous bone volume to stabilize (30). To reduce the possibility of effects of androgen treatment being due to aromatization, we used the nonaromatizable 5 α -dihydrotestosterone (DHT; see Ref. 16).

MATERIALS AND METHODS

Female Wistar rats (13 wk old) obtained from the St. George's Hospital Medical School stock were OVX under halothane anesthesia using a dorsal approach or subjected to sham ovariectomy (sham). They were then divided into one group of seven sham animals and five groups of seven OVX animals and housed at 21°C with a 12:12-h light-dark cycle. Food (Rat-mouse Diet I; Special Diet Services, Witham, Essex, UK) and water were available ad libitum. DHT (Sigma, Poole, Dorset, UK) was dissolved in 5% benzyl alcohol (Sigma) and 95% corn oil (Sigma). DHT [0 (i.e., vehicle alone), 0.01, 0.1, or 1.0 mg/kg] was then given by daily subcutaneous injection for 60 days, delaying treatment until 90 days after ovariectomy. To provide a pretreatment baseline value for cancellous bone volume (BV/TV), one group of OVX animals was killed 90 days after ovariectomy, before the start of the 60-day treatment period. Tetracycline hydrochloride (25 mg/kg; Lederle Laboratory, Gosport, Hants, UK) and calcein (30 mg/kg; Sigma) were administered intraperitoneally 1 and 5 days before the end of the experiment, respectively. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

At the termination of the study, animals were weighed, bled by vena caval puncture under halothane anesthesia, and then killed by cervical dislocation. Uteri were subsequently removed and weighed. Success of ovariectomy was also assessed by measuring the serum concentration of 17 β -estradiol by radioimmunoassay (Coat-a-Count; Diagnostic Products, Glyn Rhonwy, Gwynedd, UK). Right tibiae were subsequently freed from soft tissue, fixed in 70% alcohol for 48 h, then dehydrated through graded alcohols and embedded without decalcification in London resin (London Resin, Basingstoke, Hants, UK). Longitudinal sections of the proximal metaphysis were prepared using a Jung K microtome (Cambridge Instruments, Cambridge, Cambs, UK); 5- μ m sections were stained with toluidine blue, and 14- μ m unstained sections were prepared for fluorescence microscopy. Bone histomorphometry was performed using transmitted and epifluorescent light microscopy linked to a computer-assisted image analyzer (Perceptive Instruments, Halstead, Essex, UK). Bone volume and surface parameters were measured by tracing relevant features with a cursor on the video screen image. All sections were examined blind.

BV/TV was assessed at the proximal tibial metaphysis on two nonconsecutive toluidine blue-stained sections for each animal. The standard area of 2.5 mm² employed, which was examined at $\times 25$ magnification, was situated 1 mm from the

Table 1. Effect of 5 α -dihydrotestosterone on body and uterine weight and serum 17 β -estradiol

Group	Body Wt, g	Uterine Wt, g	17 β -Estradiol, pg/ml	Final Age, days
Sham	338 \pm 12	0.61 \pm 0.06	36.8 \pm 12.3	240
OVX 90 days	393 \pm 10*	0.16 \pm 0.01*		180
DHT, mg·kg ⁻¹ ·day ⁻¹				
0	409 \pm 15*	0.12 \pm 0.01*	13.5 \pm 2.1*	240
0.01	425 \pm 15*	0.12 \pm 0.01*	12.9 \pm 2.3*	240
0.1	454 \pm 27†	0.12 \pm 0.01*	18.3 \pm 4.6*	240
1.0	425 \pm 17*	0.47 \pm 0.02‡	15.4 \pm 2.5*	240

Results show means \pm SE for body weight, uterine weight, serum 17 β -estradiol concentration, and final age of animals in sham rats and ovariectomized (OVX) animals killed after 90 days or subsequently given 5 α -dihydrotestosterone (DHT) for 60 days. * P < 0.05 vs. sham; † P < 0.05 vs. sham and 0 DHT; ‡ P < 0.05 vs. all other groups [by analysis of variance (ANOVA)].

growth plate. This distance was chosen to exclude both the primary spongiosa (maximum width 0.5 mm) and bone formed during the course of the experiment, as estimated from the product of treatment duration (60 days) and longitudinal growth rate (LGR; maximum 7.4 μ m/day). BV/TV was expressed as percentage of tissue volume composed of bone. Trabecular thickness and number were calculated according to standard assumptions (19). Static parameters [osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and osteoclast number (NOc./BS)] were recorded at \times 160 magnification in the same area as that used for BV/TV measurements.

Fluorochrome measurements were made on two nonconsecutive unstained 14- μ m-thick sections per animal. Longitudinal bone growth was obtained from the distance between the tetracycline and calcein bands lying distal to the epiphyseal growth plate, and LGR was derived by dividing this distance by the time interval between the administration of these two labels. Fluorochrome-labeled trabecular surfaces were measured at \times 160 magnification throughout a corresponding area to that used for assessing bone volume. The proportion of trabecular surface covered by double fluorochrome label (dLS/BS) was recorded, as was the mineral apposition rate (MAR), the latter being obtained by dividing the interlabel distance by the time interval between label administration. The bone formation rate (BFR/BS; tissue level, total surface referent) was obtained from the product of dLS/BS and MAR. Values for the apposition rate were not corrected for the obliquity of the plane of section of cancellous bone.

Cross sections of the tibial diaphysis, consisting of 14- μ m unstained sections taken immediately proximal to the tibiofibular anastomosis, were obtained for assessment of cortical parameters. The latter were assessed on two unstained diaphysis cross sections per animal as follows: total bone area, medullary area, cortical bone area (total bone area - medullary area), periosteal dLS/BS, MAR, and BFR/BS, and endocortical dLS/BS. Results from the histomorphometric analysis are expressed as the means \pm SE. Statistical analysis was by Fisher's least-significant difference method for multiple comparisons in a one-way analysis of variance. Tests were carried out with Statview 4.0 (Abacus Concepts, Cupertino, CA). Statistical significance was considered at P < 0.05.

RESULTS

As expected, ovariectomy led to an increase in body weight (Table 1). Body weight was also significantly greater in OVX animals treated with 0.1 mg/kg DHT

compared with OVX animals given vehicle alone. Results for uterine weight confirmed ovariectomy. In addition, 1.0 mg/kg DHT was found to increase uterine weight to an intermediate value between sham and OVX animals, consistent with a previous report that DHT exerts a uterotrophic effect at high doses (21). Serum concentration of 17 β -estradiol fell significantly after ovariectomy and was unaffected by treatment with DHT.

Ovariectomy led to a marked decrease in BV/TV (Fig. 1 and Table 2) due to a reduction in the number and thickness of trabeculae (Figs. 2, A and B). Although indexes of cancellous bone formation and resorption were not significantly different between OVX rats given vehicle alone and sham animals, our results are consistent with bone turnover remaining elevated, albeit to a modest extent, by termination of the experiment 150 days after ovariectomy (Tables 2 and 3). Age itself had little effect on cancellous bone formation and resorption, as judged by comparing these indexes in OVX animals killed 90 days after ovariectomy with those subsequently treated with vehicle for 60 days. In contrast, OVX rats showed significant age-related decreases in longitudinal growth and periosteal and endocortical bone formation (Fig. 3 and Table 4).

Treatment of OVX animals with 0.01, 0.1, and 1.0 mg/kg DHT for 60 days was associated with a greater BV/TV than OVX animals either killed 90 days after ovariectomy or subsequently treated with vehicle alone for 60 days (Fig. 1 and Table 2). This increase in BV/TV after treatment with DHT was partly caused by a dose-responsive increase in trabecular number (Fig. 2A). However, only 1.0 mg/kg DHT significantly increased trabecular number compared with the pretreatment OVX group, and, in all DHT-treated groups, trabecular number remained substantially below that of sham-operated animals. Our results also suggested that an increase in trabecular thickness had contributed to the gain in BV/TV after treatment of OVX animals with DHT (Fig. 2B). In contrast to the effect of DHT on trabecular number, all concentrations of DHT significantly increased trabecular thickness compared with the pretreatment OVX group, with this being restored, if anything, to above that of sham-operated animals.

Static and dynamic indexes of cancellous bone formation were similar in OVX animals given DHT or vehicle alone (Tables 2 and 3). However, the increase in dLS/BS and BFR/BS in OVX animals treated with 0.01 and 0.1 mg/kg DHT reached significance compared with sham animals (Table 3). Although 0.01 and 0.1 mg/kg DHT had no significant effect on cancellous bone osteoclast indexes, these were reduced by 1.0 mg/kg DHT (Oc.S/BS and NOc./BS; Table 2).

DHT was also found to cause dose-responsive stimulation of LGR (Fig. 3) and of periosteal MAR, dLS/BS, and BFR/BS (Table 4). In addition, 0.01 and 0.1 mg/kg DHT increased endosteal dLS/BS. In contrast, 1.0 mg/kg DHT suppressed endocortical dLS/BS, presumably reflecting the tendency of this dose to suppress bone turnover. Despite these effects on cortical bone formation, DHT had little effect on diaphyseal dimensions

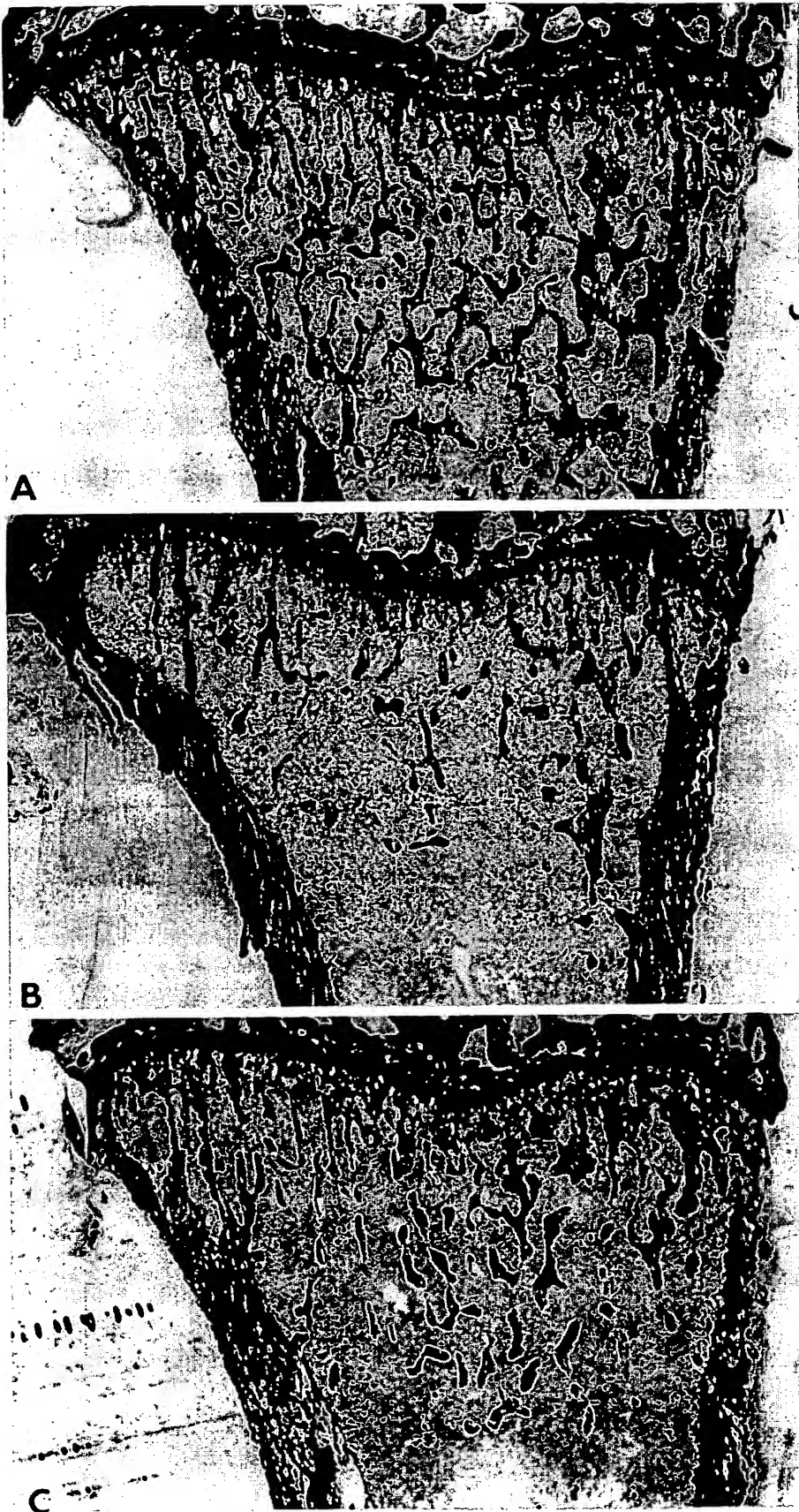


Fig. 1. Longitudinal sections of proximal tibial metaphysis stained with toluidine blue from sham rat (A), ovariectomized (OVX) rat given vehicle alone (B), and OVX rat given 1.0 mg/kg 5 α -dihydrotestosterone (C; magnification $\times 25$).

Table 2. Effect of 5 α -dihydrotestosterone on cancellous static histomorphometric parameters

Group	BV/TV, %	Ob.S/BS, %	Oc.S/BS, %	NOc./BS, n/mm
Sham	33.1 \pm 1.6	0.62 \pm 0.11	0.16 \pm 0.04	0.06 \pm 0.01
OVX 90 days	10.4 \pm 1.8*	1.37 \pm 0.39	0.23 \pm 0.04	0.10 \pm 0.02
DHT, mg· kg ⁻¹ ·day ⁻¹				
0	9.4 \pm 1.8*	1.16 \pm 0.36	0.26 \pm 0.06	0.12 \pm 0.03
0.01	15.7 \pm 2.7†	1.32 \pm 0.55	0.23 \pm 0.05	0.10 \pm 0.03
0.1	17.0 \pm 1.6†	1.09 \pm 0.20	0.29 \pm 0.06	0.12 \pm 0.03
1.0	18.8 \pm 1.2†	0.83 \pm 0.16	0.08 \pm 0.07‡	0.04 \pm 0.03§

Results show means \pm SE for cancellous bone volume (BV/TV), osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and osteoclast number (NOc./BS) at proximal tibial metaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham; † P < 0.05 vs. sham, OVX 90 days, and 0 DHT; ‡ P < 0.05 vs. OVX 90 days, 0 DHT, and 0.1 DHT; § P < 0.05 vs. 0 and 0.1 DHT (by ANOVA).

over the duration of the study (Table 5). Cross-sectional and cortical areas were found to be greater in OVX animals than in sham animals when results from OVX groups were pooled (see Table 5), consistent with previous reports of minor changes in diaphyseal area after ovariectomy in the rat (26).

DISCUSSION

We have found that DHT partially restores cancellous bone volume in female rats rendered osteopenic by ovariectomy. This increase reflected a net gain in bone volume, rather than the prevention of further bone loss, since DHT also increased bone volume compared with OVX rats killed immediately before the start of DHT administration. In previous studies of the effects of androgens in the rat, orchidectomy was noted to cause loss of cancellous bone in males (25, 27–29), which was prevented by giving androgens such as DHT (28). In addition, a microdensitometric study found that the anabolic steroid nandrolone decanoate increased bone mineral content in osteopenic OVX rats, although this effect was limited to the diaphysis (20). However, microdensitometry does not accurately distinguish trabecular from cortical bone and does not enable the histodynamic assessment of bone formation and resorption. To our knowledge, there have been no previous histomorphometric assessments of the effects of androgens in female rats.

The increase in bone volume after DHT administration appeared to be due to an increase in both trabecular number and thickness. However, the effect on trabecular thickness appeared stronger, since this was increased by all doses of DHT. Furthermore, DHT restored trabecular thickness, but not number, to that of sham-operated animals. In fact, it seems likely that agents that are able to increase cancellous bone volume of the human skeleton do so by enlarging existing trabeculae, rather than by causing their formation *de novo*. If so, the increase in trabecular thickness that we have found after treatment of OVX rats with androgens may accurately reflect the mechanism of increase in bone mass previously observed in women treated with

anabolic steroids (6, 11, 17). However, there have been no bone biopsy studies that address the effects of these agents on trabecular architecture in postmenopausal women.

Cancellous bone formation rate was significantly increased compared with sham rats in OVX animals treated with 0.01 and 0.1 mg/kg DHT, but not those given vehicle alone, consistent with additive effects of ovariectomy and DHT on cancellous bone formation. This suggests that DHT may have increased cancellous bone volume in osteopenic OVX rats at least in part by stimulating bone formation. However, because indexes of cancellous bone formation in vehicle- and DHT-treated OVX rats were not significantly different, it remains unclear as to whether cancellous bone forma-

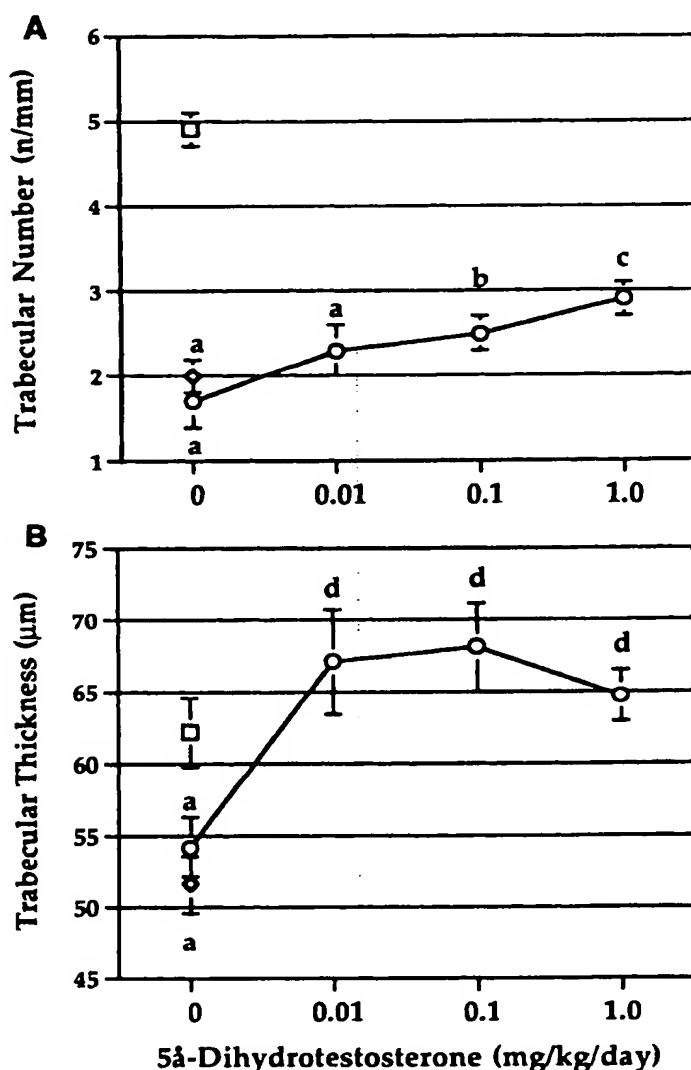


Fig. 2. Results show means \pm SE trabecular number (A) and trabecular thickness (B) at proximal tibial metaphysis in sham (□) and OVX animals killed 90 days after ovariectomy (○; OVX 90 days) or subsequently given 5 α -dihydrotestosterone (DHT) for 60 days (○). * P < 0.05 vs. sham; † P < 0.05 vs. sham and 0 DHT; ‡ P < 0.05 vs. sham, OVX 90 days, and 0 DHT; § P < 0.05 vs. OVX 90 days and 0 DHT (by analysis of variance (ANOVA)).

Table 3. Effect of 5 α -dihydrotestosterone on cancellous dynamic histomorphometric parameters

Group	dLS/BS, %	MAR, $\mu\text{m}/\text{day}$	BFR/BS, $10^{-2} \mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{day}^{-1}$
Sham	6.4 \pm 1.1	1.09 \pm 0.05	7.1 \pm 1.3
OVX 90 days	11.9 \pm 3.0	1.10 \pm 0.06	13.9 \pm 3.9
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$			
0	12.1 \pm 2.8	1.16 \pm 0.06	14.6 \pm 3.7
0.01	16.0 \pm 1.9*	1.21 \pm 0.05	19.5 \pm 2.7*
0.1	16.9 \pm 3.6*	1.22 \pm 0.03	20.9 \pm 4.9*
1.0	11.4 \pm 2.3	1.18 \pm 0.04	13.4 \pm 2.5

Results show means \pm SE of double-labeled surface (dLS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) at proximal tibial metaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham (by ANOVA).

tion was in fact stimulated by DHT. In contrast, at the growth plate, endocortical, and periosteal surfaces, a significant stimulatory effect of DHT on bone formation was observed, raising the possibility that DHT exerts an anabolic effect on the skeleton in general. Although these increases in cortical bone formation had no effect on cortical area over the study period, this may have reflected an insufficient duration of treatment for alterations in cortical, as well as cancellous, bone mass to occur.

Suppression of bone resorption may have contributed to the increase in cancellous bone volume after treatment with DHT since, although 0.01 and 0.1 mg/kg DHT had no significant effect on bone resorption, as assessed by osteoclast surface and number, 1.0 mg/kg DHT was found to reduce these resorption parameters. Interestingly, in contrast to lower concentrations, the same dose of DHT also suppressed endocortical bone formation and did not increase cancellous bone forma-

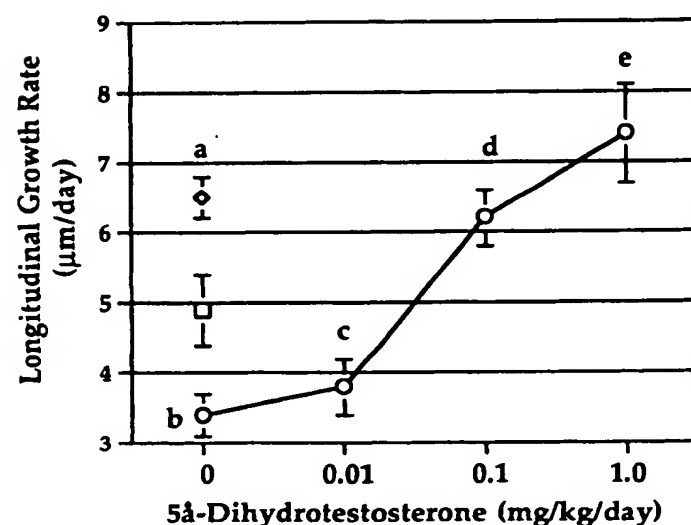


Fig. 3. Results show means \pm SE longitudinal growth rate in sham \square and OVX animals killed 90 days after ovariectomy (\circ) or subsequently given DHT for 60 days (\circ). * P < 0.05 vs. sham; ^b P < 0.05 vs. sham and OVX 90 days; ^c P < 0.05 vs. OVX 90 days; ^d P < 0.05 vs. 0 and 0.01 DHT; ^e P < 0.05 vs. sham and 0 and 0.01 DHT (by ANOVA).

Table 4. Effect of 5 α -dihydrotestosterone on dynamic cortical histomorphometric parameters

Group	Periosteal dLS/BS, %	Periosteal MAR, $\mu\text{m}/\text{day}$	Periosteal BFR/BS, $10^{-2} \mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{day}^{-1}$	Endocortical dLS/BS, %
Sham	2.0 \pm 1.7	0.75 \pm 0.16	2.0 \pm 1.8	0.4 \pm 0.4
OVX 90 days	11.1 \pm 2.1*	0.90 \pm 0.07†	10.4 \pm 2.4*	4.1 \pm 1.9‡
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$				
0	2.8 \pm 2.0	0.51 \pm 0.04	1.3 \pm 0.8	1.5 \pm 0.8
0.01	4.6 \pm 2.9	0.76 \pm 0.14	4.4 \pm 3.0	5.5 \pm 1.5*
0.1	5.1 \pm 1.5	0.88 \pm 0.08†	4.4 \pm 1.2	4.8 \pm 1.8*
1.0	11.1 \pm 5.1*	0.89 \pm 0.07†	10.2 \pm 4.4*	0.1 \pm 0.1§

Results show means \pm SE of periosteal dLS/BS, MAR, and BFR/BS and endocortical dLS/BS at tibial diaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. * P < 0.05 vs. sham and 0 DHT; † P < 0.05 vs. 0 DHT; ‡ P < 0.05 vs. sham; § P < 0.05 vs. 0.01 and 0.1 DHT (by ANOVA).

tion compared with sham animals. Therefore, inhibition of bone resorption by 1.0 mg/kg DHT may have led to a reduction in bone formation at sites where bone formation is coupled to resorption, as occurs with other inhibitors of bone resorption such as bisphosphonates, 17 β -estradiol, and calcitonin (31, 32). Hence, DHT appears to influence skeletal metabolism in osteopenic OVX rats both by stimulating bone formation and suppressing resorption, although it is unclear which, if any, of these actions predominate at cancellous sites.

These actions of DHT on skeletal metabolism are consistent with previous reports that, in male rats and humans, androgens both stimulate bone formation (1, 10, 25) and suppress bone resorption (24, 27, 28). Because DHT shows high-affinity binding with classical androgen receptors (15), which bone cells have been found to possess (7), these actions of DHT may be mediated by skeletal androgen receptors. Moreover, unlike other androgens, DHT is not thought to undergo peripheral aromatization to estrogen (16), consistent with our observation that estrogen levels were similar in OVX animals receiving DHT and vehicle alone. The actions of DHT on bone and cartilage that we and others have described are also consistent with the effects of DHT on isolated bone cell cultures. For example, DHT has

Table 5. Effect of 5 α -dihydrotestosterone on cortical areas

Group	Cross-sectional Area, mm^2	Medullary Area, mm^2	Cortical Area, mm^2
Sham	4.39 \pm 0.17	0.71 \pm 0.05	3.68 \pm 0.13
OVX 90 days	4.58 \pm 0.06	0.68 \pm 0.03	3.90 \pm 0.07
DHT, $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$			
0	4.79 \pm 0.06	0.76 \pm 0.06	4.03 \pm 0.08
0.01	4.90 \pm 0.18	0.83 \pm 0.04	4.07 \pm 0.17
0.1	4.79 \pm 0.25	0.77 \pm 0.08	4.02 \pm 0.18
1.0	4.76 \pm 0.11	0.76 \pm 0.05	3.99 \pm 0.12
OVX combined	4.81 \pm 0.17*	0.78 \pm 0.03	4.03 \pm 0.07*

Results show means \pm SE of cross-sectional, medullary, and cortical areas at tibial diaphysis in sham animals and OVX rats killed after 90 days or subsequently given DHT for 60 days. OVX combined was obtained by pooling the results from all 240-day-old OVX animals. * P < 0.05 vs. sham (unpaired 2-tailed Student's t -test).

been found to stimulate the proliferation of isolated osteoblasts (14), to increase chondrocyte DNA synthesis (4), and to suppress the bone resorptive activity of isolated osteoclasts (18).

Our findings, which suggest that relatively low doses of DHT stimulate bone formation in female rats, raise the possibility that androgens play a physiological role in regulating bone formation in females. This is consistent with the observation that the antiandrogen flutamide reduced total body calcium in female rats by suppressing bone formation (12). However, we only found an antiresorptive action of DHT at the highest dose, suggesting that androgens may not influence bone resorption in female rats under physiological conditions. In contrast, studies in orchidectomized rats suggest that physiological concentrations of androgens both stimulate bone formation and suppress resorption in male rats (25, 27). This suggests that there may be certain sex differences in responsiveness of the skeleton to androgens, which is consistent with the observation that other skeletal effects of androgens, such as stimulation of diaphyseal creatine kinase activity and DNA synthesis, are also gender specific (23).

The ability of DHT to increase cancellous bone in osteopenic OVX rats does not appear to be shared by other sex steroids such as estrogen. For example, although estrogen prevents ovariectomy-induced bone loss, it fails to significantly increase cancellous bone volume in osteopenic animals (22). These results might suggest that anabolic steroids derived from androgens are more likely to be effective at reversing bone loss in patients with postmenopausal osteoporosis. However, although androgen-related steroids have been found to increase bone mass in these patients, whether this represents an advantage over the skeletal response to estrogen therapy is currently unknown.

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A Prospective Randomized Trial Comparing Low Dose Flutamide, Finasteride, Ketoconazole, and Cyproterone Acetate-Estrogen Regimens in the Treatment of Hirsutism

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ABSTRACT

Sixty-six hirsute women were randomized and treated with 1) flutamide (n = 15), 250 mg/day; 2) finasteride (n = 15), 5 mg/day; 3) ketoconazole (n = 16), 300 mg/day; and 4) ethinyl estradiol (EE)-cyproterone acetate (CPA; n = 20), 0.01 mg EE/day for the first week, 0.02 mg EE/day for the second week, and 0.01 mg EE/day for the third week, followed by a pause of 7 days, then 12.5 mg CPA/day added during the first 10 days of every month for 12 months. Hirsutism was evaluated by the Ferriman-Gallwey score, and hair diameter and hair growth rate were determined by a special image analysis processor in basal conditions and after 90, 180, 270, and 360 days of treatment. All treatments produced a significant decrease in the hirsutism score, hair diameter, and daily hair growth rate: flutamide, $-55 \pm 13\%$, $-21 \pm 14\%$, and $-37 \pm 18\%$; finasteride, $-44 \pm 13\%$, $-16 \pm 12\%$, and $-27 \pm 14\%$; ketoconazole, $-53 \pm 18\%$, $-14 \pm 12\%$, and $-30 \pm 21\%$; and EE-CPA, $-60 \pm 18\%$, $-20 \pm 11\%$, and $-28 \pm 21\%$.

Some differences existed among treatments with regard to effectiveness; EE-CPA and flutamide seem to be the most efficacious in improving hirsutism. For the hirsutism score, a greater decrease was seen with EE-CPA ($-60 \pm 18\%$) than with finasteride ($-44 \pm 13\%$; $P < 0.01$) and a greater decrease was seen with flutamide ($-58 \pm 18\%$) than with finasteride ($-44 \pm 13\%$; $P < 0.05$). Flutamide is the fastest in decreasing hair diameter; EE-CPA is the fastest in slowing down

hair growth, even though at the end of the treatment there was a significant difference between flutamide and finasteride only ($-41 \pm 18\%$ vs. $-27 \pm 14\%$; $P < 0.05$).

Flutamide, ketoconazole, and EE-CPA induced a significant decrease in total and free testosterone, 5 α -dihydrotestosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione plasma levels. During the EE-CPA treatment, gonadotropins were suppressed, and the sex hormone-binding globulin level increased. Finasteride induced a decrease in dehydroepiandrosterone sulfate and 5 α -dihydrotestosterone and an increase in testosterone levels.

Very few side-effects were observed during treatment with low doses of flutamide, EE-CPA, and particularly finasteride. Flutamide induced a decrease whereas EE-CPA induced an increase in triglycerides and cholesterol, showing higher values within the normal range. Ketoconazole induced several side-effects and complications, and several people dropped out of the study.

Despite different modalities of action and significantly different effects on androgen levels, low doses of flutamide, finasteride, and EE-CPA constitute very satisfactory alternative therapeutic regimens in the treatment of hirsutism. (*J Clin Endocrinol Metab* 84: 1304-1310, 1999)

FLUTAMIDE (1-5), finasteride (6-10), ketoconazole (11-14), and cyproterone acetate (CPA) (15-20) are commonly employed in the treatment of hirsutism. Different therapeutic regimens have been used successfully; however, only a few randomized controlled trials exist, and subjective methods of evaluation are generally employed.

The aim of the present report was to compare, in a prospective, comparative, randomized study, as objectively as possible, the therapeutic efficacy as well as the endocrine and metabolic effects and reliability of low dose regimens of flutamide, finasteride, ketoconazole, and a combination of CPA and ethinyl estradiol (EE).

Subjects and Methods

Sixty-six premenopausal hirsute women (mean \pm SD age, 22.9 ± 4.7) were referred to the Reproductive Medicine Unit of the University of

Bologna (Bologna, Italy) for evaluation and treatment of hirsutism. The mean \pm SD body weight was 61 ± 10 kg, and the mean \pm SD height was 163 ± 6 cm. The mean body mass index (BMI) was 22.7 ± 2.7 (normal range, 18-24); 11 subjects (16%) were overweight (mean BMI, 27 ± 2.6).

Regular menses were reported by 29 of the 66 women; 32 had oligomenorrhea, 3 had amenorrhea, and 2 had polymenorrhea. Thirty-eight patients (58%) had ovulatory cycles (on the basis of typical progesterone levels in the premenstrual phase), and 28 (42%) had anovulatory cycles. Each patient underwent a complete medical and gynecological examination. In accordance with our codified parameters (21), all subjects had an etiological diagnosis of hirsutism. None of the women gave evidence of a hormonally active adrenal gland, an ovarian tumor, or Cushing's, PRL, or thyroid disorder. Twenty-seven patients (41%) had a diagnosis of polycystic ovary syndrome; 18 had anovulatory or oligoovulatory cycles, elevated plasma LH concentrations (LH/FSH ratio >2), high levels of testosterone and androstenedione, and echographic evidence of enlarged polycystic ovaries. Nine patients had the concomitant presence of high dehydroepiandrosterone sulfate levels. Fourteen hirsute patients (21%) suffered from a mild form of nonclassic adrenal hyperplasia with high 17 α -hydroxyprogesterone values, as diagnosed by ACTH test (21). Twenty-five patients (38%) were classified as having idiopathic hirsutism because they did not present any of the clinical features found in the other groups and had ovulatory cycles. In the entire population studied the hirsutism score ranged from 7-22.

Patients were randomized into four groups for treatment, indepen-

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dently of the diagnosis made. No significant differences were observed between groups regarding the prevalence of the diagnosis of hirsutism, clinical parameters, and menstrual rhythm or ovulatory/anovulatory cycles; thus, the patients were randomized into four comparable groups. Group 1 ($n = 15$; age, 22.6 ± 4 yr; BMI, 22.9 ± 2 kg/m²) received 250 mg/day flutamide, group 2 ($n = 15$; age, 23.2 ± 3 yr; BMI, 22.5 ± 3 kg/m²) received 5 mg/day finasteride, group 3 ($n = 16$; age, 23.2 ± 5 yr; BMI, 23.0 ± 3 kg/m²) received 300 mg/day ketoconazole, and group 4 ($n = 20$; age, 22.9 ± 4 yr; BMI, 22.6 ± 2 kg/m²) received a treatment regimen with low EE and CPA doses (the patients received 0.01 mg EE/day for the first week, 0.02 mg EE/day for the second week, and 0.01 mg EE/day for the third week, followed by a pause of 7 days, then 12.5 mg CPA/day administered in a reverse sequential regimen during the first 10 days of each treatment cycle). We planned a 12-month therapy period, and either the barrier method or intrauterine contraception was employed during the study in sexually active women to avoid any risk of conception.

Clinical and hormonal controls were performed in basal conditions and after 3, 6, 9, and 12 months. Each woman was studied during the early follicular phase of her menstrual cycle (3–6 days after the onset of a spontaneous menstrual flow) when present or at random in amenorrheic patients in basal conditions and after 180 and 360 days of treatment.

The study was approved by the ethical committee of the Institute of Obstetrics and Gynecology of the University of Bologna, and informed consent was obtained from each patient. All procedures followed in this study were in accordance with the Helsinki Declaration of 1975.

Hirsutism evaluation, clinical side-effects, endocrine and biochemical parameters [blood glucose, cholesterol, high density lipoprotein cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyltransferase, alkaline phosphatase, bilirubin, antithrombin III, and fibrinogen] were determined at each control visit.

Hirsutism and hair growth evaluation

Hirsutism was evaluated with a 2-fold criteria of control so as to have a method of analysis as objective as possible. 1) Hirsutism grading was codified by calculating the hirsutism score according to the modified Ferriman and Gallwey method (22), and the normal range was considered to be no higher than 8. Two patients with a total score of 7 were included because a severe regional (upper lip and thigh) hair growth, and the entry criteria for all patients was a score of 7 or greater. 2) As previously reported (12), hair parameters were codified using an IBAS image analyzer (Kontron Bildanalyse GmbH, Munich, Germany), a special image analysis processor with a sensitivity of 0.001 mm. First, all patients were shaved in a prefixed area of the right thigh. After 90 days, at least 20 hairs (basal hair growth) were cut from the same thigh area using curved scissors; then the area was reshaved, and treatments were started. This same procedure was repeated after 90, 180, 270, and 360 days of therapy. The mean diameter, hair length, and daily growth rate (obtained by dividing the length of each hair by the number of the days elapsed between shaving and the subsequent cut) were estimated. Considering that anagen hair growth on the thigh has a mean duration of 22 days and that the complete hair cycle is 84 days (23), a 90-day period of basal observation seems to be correct for establishing the mean basal hair growth in each group.

Self-reported evaluation

Patients' self-evaluation of the clinical outcome of the treatment was obtained. Each patient rated his appreciation as dissatisfied, satisfied, or highly satisfied.

Hormone assay

Blood samples were drawn at 0800 h; all samples from each subject were run in the same RIA. The RIA techniques used for hormonal measurements were: gonadotropins (FSH-LH) and PRL, rapid double antibody (kits purchased from Biodata, Rome, Italy); 17-hydroxyprogesterone (17-P) and progesterone (P), chromatographic separation on Sephadex LH-20 columns; dehydroepiandrosterone sulfate (DHAS) performed directly on diluted plasma, testosterone (T), 5 α -dehydrotestosterone (DHT), androstenedione (A), and 17 β -estradiol (E₂), TLC on silica

gel 60 F254; dehydroepiandrosterone (DHA), plasma extraction with ethyl ether, as previously described (24), using an ACTH kit purchased from CIS (Gif-Sur-Yvette, France) and a cortisol (F) kit purchased from Diagnostic Systems Laboratories (Webster, TX); free testosterone (Tf), the Coat-A-Count free testosterone procedure of Diagnostic Products (Los Angeles, CA); and sex hormone-binding globulin (SHBG), non-competitive liquid phase immunoradiometric assay (Farnos Diagnostic, Oulunsalo, Finland).

Statistical analysis

Paired and unpaired Student's *t* tests and ANOVA were used for statistical analysis, as needed. Values are expressed as the mean \pm SD.

Results

Hirsutism

Figure 1 shows the results of the different regimens of therapy during the entire treatment period. In each subject who finished the study, not less than 100 hairs were analyzed by IBAS, both under basal conditions and during the entire cycle of treatment. Under basal conditions, 1848 hairs were analyzed, and 6800 hairs were analyzed during the entire cycle of treatment.

In group 1 (flutamide), hirsutism improved in all subjects. The mean basal score (Fig. 1; 14.2 ± 4.5) progressively decreased and dropped to 6.4 ± 3.5 ($P < 0.001$; $-55 \pm 13\%$) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.169 ± 0.02 to 0.133 ± 0.02 mm ($P < 0.001$; $-21 \pm 14\%$), and the mean daily rate of hair growth (Fig. 1) fell progressively from 0.153 ± 0.03 to 0.084 ± 0.04 mm/day ($P < 0.001$; $-41 \pm 18\%$).

In group 2 (finasteride), hirsutism improved in all subjects. The mean basal score (Fig. 1; 12.4 ± 4.8) slowly dropped during therapy to 6.9 ± 3.5 ($P < 0.02$; $-44 \pm 13\%$) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.174 ± 0.02 to 0.147 ± 0.02 mm ($P < 0.001$; $-16 \pm 12\%$), and the mean daily rate (Fig. 1) of hair growth fell from 0.127 ± 0.05 to 0.095 ± 0.04 mm/day ($P < 0.005$; $-27 \pm 14\%$).

In group 3 (ketoconazole), 8 of 16 subjects who started the therapy stopped taking the drug within 180 days because of several side-effects and complications; hirsutism had improved in the 8 subjects who concluded the 12-month therapy period (mean basal score, 13.8 ± 4.4 ; 12-month therapy score, 6.5 ± 4.8 ; $P < 0.005$; $-53 \pm 18\%$; Fig. 1). The mean diameter (Fig. 1) fell progressively from 0.177 ± 0.01 to 0.148 ± 0.02 mm ($P < 0.005$; $-14 \pm 12\%$), and the mean daily rate of hair growth (Fig. 1) fell from 0.129 ± 0.03 to 0.090 ± 0.04 mm/day ($P < 0.005$; $-30 \pm 21\%$). Six of 8 hirsute subjects who interrupted the therapy had slower hair growth during the therapy on the basis of their subjective evaluations.

In group 4 (EE-CPA), hirsutism improved in all subjects. The mean basal score (Fig. 1; 13.3 ± 5.1) dropped to 6.1 ± 5.5 ($P < 0.001$; $-60 \pm 18\%$) after 12 months of treatment. The mean diameter (Fig. 1) fell from 0.164 ± 0.05 to 0.138 ± 0.02 mm ($P < 0.001$; $-20 \pm 11\%$). The mean daily rate (Fig. 1) of hair growth decreased during the first 90 days and fell from 0.127 ± 0.05 to 0.090 ± 0.03 mm/day ($P < 0.001$) ($-28 \pm 21\%$) after 12 months.

Comparative data among groups

There were no significant differences among groups with respect to their clinical basic data, endocrine parameters

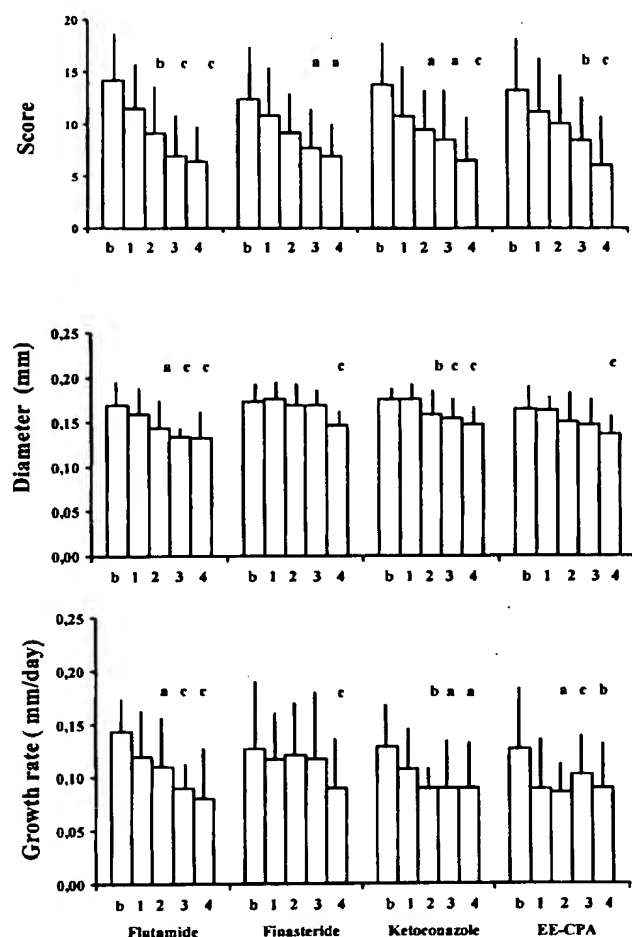


FIG. 1. Histograms representing the mean \pm SD hirsutism score, diameter, and daily growth rate of hair under basal conditions (b) and after 90 (1), 180 (2), 270 (3), and 360 (4) days of therapy with different treatment regimens. In the ketoconazole group, 2 patients of 16 dropped out during the first 90 days of therapy, and 6 patients dropped out during the first 180 days. At 180, 270, and 360 days of treatment, only 8 subjects were studied. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$ (vs. basal within each group). For hirsutism score: by ANOVA among groups in basal conditions, $P = \text{NS}$; by ANOVA among groups during treatment, $P = \text{NS}$. For hair diameter: by ANOVA among groups in basal conditions, $P = \text{NS}$; by ANOVA among groups during treatment, significant differences at 90 ($F = 3.9$; $P < 0.01$), 180 ($F = 3.9$; $P < 0.01$), and 270 ($F = 6.7$; $P > 0.01$) days. For hair growth: by ANOVA among groups in basal conditions, $P = \text{NS}$; by ANOVA among groups during treatment, significant differences at 90 ($F = 3.6$; $P < 0.02$) and 180 ($F = 5.1$; $P < 0.003$) days.

(Tables 1 and 2), hirsutism score, or basal hair characteristics (Fig. 1).

For the hirsutism score (Fig. 1), no differences (by ANOVA, $P = \text{NS}$) were observed among the groups during the control period, confirming the efficacy of all four treatments. However significantly higher differences were observed in the percentage of decrease (Fig. 2) at the end of treatment in the cases of flutamide vs. finasteride ($-55 \pm 18\%$

vs. $-44 \pm 13\%$; $P < 0.05$) and EE-CPA vs. finasteride ($-60 \pm 18\%$ vs. $-44 \pm 13\%$; $P < 0.01$).

For hair diameter (Fig. 1), significant differences were observed at 90 days ($F = 3.94$; $P < 0.01$), 180 days ($F = 3.6$; $P < 0.01$), and 270 days ($F = 6.7$; $P < 0.001$) among the groups. Flutamide induced the quickest decrease in hair diameter during treatment (at 90 days of treatment) even though the differences in the percent decrease disappeared at the end of treatment (Fig. 2).

For the hair growth rate (Figs. 1 and 2), significant differences among groups were observed at 90 days ($F = 3.6$; $P < 0.02$) and 180 days ($F = 5.1$; $P < 0.003$). Finasteride was the slowest in decreasing hair growth, whereas EE-CPA was the quickest. Flutamide acted progressively, and there was a significant difference between flutamide and finasteride ($-41 \pm 18\%$ vs. $-27 \pm 14\%$; $P < 0.05$) at the end of treatment (Fig. 2).

Hormone concentrations

Tables 1 and 2 show the mean hormonal values under basal conditions and during the different treatments. In group 1, flutamide induced a significant decrease in 17-P, Tf, T, DHT, A, DHAS, DHA, and F and an increase in E_2 levels. In group 2, finasteride induced a significant decrease in DHAS, E_2 , and DHT mean values and an increase in T and Tf mean values. In group 3, the mean plasma levels of T, Tf, DHT, DHA, DHAS, and A decreased progressively during ketoconazole treatment, whereas FSH, LH, E_2 , F, ACTH, and 17-P values increased. In group 4 during treatment with EE/CPA, there was a decrease in LH, E_2 , 17-P, T, Tf, A, DHA, and DHAS and an increase in F and SHBG plasma levels.

Side-effects, complications, and clinical and biochemical changes (Table 3)

In group 1, only a few, transient and slight side-effects occurred, and all subjects concluded the period of treatment. After 6 months of treatment, cholesterol (-11%) and triglycerides (-22%) values dropped significantly, with respect to the basal values. No changes were observed in the other parameters.

In group 2, no side-effects, complications, or biochemical changes were observed, even though two patients were dissatisfied.

In group 3, major side-effects and complications occurred during the first 90 days of treatment. A high number of patients dropped out within the first 180 days. Mean AST, ALT, and alkaline phosphatase levels increased progressively to the upper limit of the normal adult range. Two subjects had very high AST and ALT values. Triglycerides (-25%) and cholesterol (-17%) values decreased progressively. No changes were observed in the other parameters.

In group 4, some subjects experienced a mild weight gain (<2 kg) after 6–9 months of treatment and had mild transient side-effects. One subject suffered from irregular menstrual bleeding, and one dropped out because of persistent amenorrhea during the ninth month of therapy. Cholesterol mean values increased progressively ($+21\%$) up to the upper limit of normal values (<250 mg/dL), and mean triglycerides

TABLE 1. Endocrine mean \pm SD plasma values in basal conditions and after 180 and 360 days of therapy with different treatment regimens

	LH (IU/L; normal, 2–10)	FSH (IU/L; normal, 4–10)	PRL (μ g/L; normal, 6–28)	E ₂ (pmol/L; normal, 51.4–194.5)	ACTH (pmol/L; normal, 5–60)	17P (nmol/L; normal, 0.9–3.8)	SHBG (nmol/L; normal, 16–120)
Flutamide							
A	5.3 \pm 2.6	5.5 \pm 1.0	17 \pm 6	154 \pm 44	6.9 \pm 3.1	3.3 \pm 1.2	29 \pm 11
B	4.8 \pm 1.9	5.4 \pm 0.8	18 \pm 8	243 \pm 36 ^a	5.9 \pm 2.9	2.8 \pm 1.6	34 \pm 11
C	6.0 \pm 2.4	5.5 \pm 1.0	18 \pm 10	250 \pm 33 ^a	5.5 \pm 2.7	2.2 \pm 0.8 ^b	37 \pm 6
Finasteride							
A	6.0 \pm 2.2	5.7 \pm 1.6	15 \pm 8	130 \pm 37	6.1 \pm 0.4	2.7 \pm 0.9	38 \pm 15
B	6.0 \pm 2.1	6.0 \pm 1.4	11 \pm 5	130 \pm 41	5.2 \pm 0.5	2.2 \pm 0.8	38 \pm 12
C	5.1 \pm 1.9	5.5 \pm 1.2	13 \pm 4	55 \pm 31 ^a	7 \pm 0.4	2.7 \pm 0.8	43 \pm 16
Ketoconazole							
A	5.7 \pm 2.8	4.2 \pm 1.3	16 \pm 6	180 \pm 50	3 \pm 2.1	3 \pm 1.1	26 \pm 10
B	10.2 \pm 4.7 ^c	5.4 \pm 2.1	17.3 \pm 5	280 \pm 45 ^a	7 \pm 2 ^a	5 \pm 5.3	28 \pm 11
C	12.5 \pm 5.3 ^d	6.8 \pm 2.7 ^e	17 \pm 5	270 \pm 33 ^a	6 \pm 1.9 ^d	6 \pm 3.2 ^e	27 \pm 10
EE-CPA							
A	4.7 \pm 2.1	4.8 \pm 1.2	12 \pm 5	176 \pm 52	5.4 \pm 1.8	3.2 \pm 1.1	30 \pm 11.8
B	3.0 \pm 1.6 ^b	4.9 \pm 2.0	18 \pm 10	127 \pm 62 ^e	4.8 \pm 1.6	2.2 \pm 0.9 ^d	124 \pm 45 ^a
C	3.2 \pm 1.2 ^b	4.7 \pm 1.9	17 \pm 10	92 \pm 37 ^a	4.4 \pm 1.2	1.8 \pm 0.6 ^a	113 \pm 38 ^a

Normal hormonal ranges are reported in parentheses. In the ketoconazole group, at 180, 270, and 360 days of treatment, only eight subjects were studied. A, Basal; B, 180 days; C, 360 days. Significance is given for values within the same regimen.

^a $P < 0.001$ vs. basal.

^b $P < 0.05$ vs. basal.

^c $P < 0.05$ vs. basal.

^d $P < 0.005$ vs. basal.

^e $P < 0.02$ vs. basal.

TABLE 2. Endocrine mean \pm SD plasma values in basal conditions and during treatment after 180 and 360 days of therapy with four different treatment regimens

	T _r (pmol/L; normal, 2.4–12.5)	T (nmol/L; normal, 1–3.5)	DHT (nmol/L; normal, 0.3–1.1)	DHA (nmol/L; normal, 8.5–41.2)	DHAS (μ mol/L; normal, 1.9–10.3)	A (nmol/L; normal, 1.4–15.7)	F (nmol/L; normal, 193–662)
Flutamide							
A	8 \pm 3.1	1.7 \pm 0.6	0.8 \pm 0.2	40.8 \pm 14	9 \pm 2	10.8 \pm 2.5	510 \pm 57.9
B	8.7 \pm 5.2	1.8 \pm 0.7	0.7 \pm 0.2	31 \pm 10 ^a	6 \pm 1.8 ^b	7.5 \pm 2.9 ^c	455 \pm 49.6 ^d
C	5.8 \pm 2.7 ^a	1.1 \pm 0.2 ^b	0.6 \pm 0.1 ^c	26.7 \pm 10.7 ^c	5.9 \pm 1.8 ^b	7.4 \pm 1.6 ^a	480 \pm 55.1
Finasteride							
A	5.5 \pm 2.4	1.7 \pm 0.2	0.7 \pm 0.2	37.7 \pm 21.1	9 \pm 2	7.9 \pm 2.1	474 \pm 63
B	6.5 \pm 2.4	2.0 \pm 0.2 ^b	0.6 \pm 0.2	38.1 \pm 12.1	7 \pm 1.9 ^d	9 \pm 2.3	469 \pm 77
C	7.4 \pm 2.4 ^a	2.3 \pm 0.2 ^b	0.5 \pm 0.1 ^c	25.3 \pm 12.9	7 \pm 1.9 ^d	9.2 \pm 2.5	496 \pm 74
Ketoconazole							
A	7 \pm 2	1.6 \pm 0.7	0.7 \pm 0.3	32.1 \pm 14.5	9 \pm 2	7.5 \pm 3	500 \pm 200
B	5 \pm 3	1.2 \pm 0.2 ^b	0.5 \pm 0.2	20.5 \pm 8 ^a	7.5 \pm 2.1	4.4 \pm 3.1 ^a	850 \pm 197 ^b
C	5 \pm 2 ^a	1.2 \pm 0.1 ^b	0.4 \pm 0.1 ^c	20.6 \pm 7.5 ^a	6.2 \pm 1.9 ^c	4.4 \pm 2.7 ^a	800 \pm 255 ^d
EE-CPA							
A	5.8 \pm 2.4	1.8 \pm 0.5	0.7 \pm 0.3	35 \pm 15.2	9 \pm 1.8	9.1 \pm 2.2	518 \pm 102
B	4.1 \pm 2.0 ^c	1.1 \pm 0.5 ^b	0.8 \pm 0.3	29.1 \pm 15.4	7 \pm 2 ^c	7.1 \pm 2.7 ^e	596 \pm 80 ^c
C	3.8 \pm 1.7 ^c	1.3 \pm 0.5 ^c	0.8 \pm 0.3	19.4 \pm 6.5 ^b	6 \pm 2 ^b	6.4 \pm 1.8 ^b	626 \pm 55.1 ^b

A, Basal; B, 180 days; C, 360 days. See Table 1 legend. Significance is given for values within the same regimen.

^a $P < 0.05$ vs. basal.

^b $P < 0.001$ vs. basal.

^c $P < 0.005$ vs. basal.

^d $P < 0.01$ vs. basal.

^e $P < 0.02$ vs. basal.

values increased progressively (+38%), but they remained within the normal range (20–175 mg/dL).

Discussion

A very long treatment period is always required to improve hirsutism and prevent or delay its relapse; the use, as much as possible, of low doses of antiandrogens may be a suitable choice in an attempt to prevent the incidence of side-effects and complications and to maintain treatment. The present study confirms the effectiveness of all four antiandrogens, flutamide, finasteride, ketoconazole, and cypro-

terone acetate, in the treatment of hirsutism, even if given in very low doses; however, some differences do exist.

Low as well as high doses of flutamide alone (4, 25) were effective. The hirsutism score decreased progressively (–55%), and the improvement of hirsutism was associated with a rapid decrease in hair diameter (–11% after 3 months) and a progressive decrease in the daily hair growth rate (–41% after 12 months). A net decrease in T, T_r, DHT, A, 17-P, DHA, and DHAS plasma levels during treatment was observed, in agreement with some reports (26, 27) and in disagreement with others (1, 2, 5, 28), suggesting that the

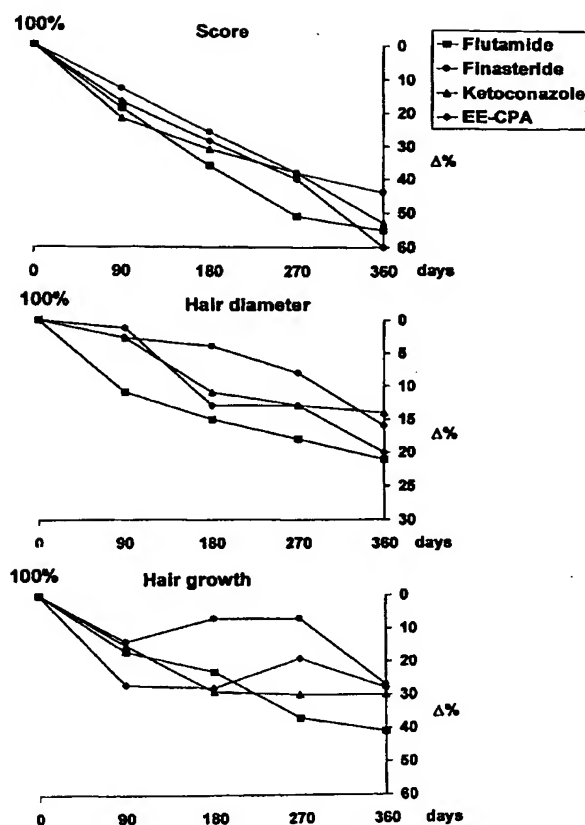


FIG. 2. Mean decreases in hirsutism score, hair diameter, and hair growth rate detected during the different treatments. Decreases are expressed as percentages with respect to basal values. For score: by ANOVA among groups during treatment, significant differences at 360 days ($F = 3.1$; $P < 0.03$); maximum change in the percent flutamide vs. finasteride after 360 days, $P < 0.05$; maximum change in the percent EE-CPA vs. finasteride after 360 days, $P < 0.01$. For hair diameter: by ANOVA among groups during treatment, significant differences at 90 ($F = 8.6$; $P < 0.001$) and 180 ($F = 3.1$; $P < 0.03$) days. For hair growth: by ANOVA among groups during treatment, significant differences at 90 ($F = 3.1$; $P < 0.03$), 180 ($F = 5.2$; $P < 0.003$), and 270 ($F = 9.9$; $P < 0.001$) days. For maximum change in percent flutamide vs. finasteride after 360 days, $P < 0.05$.

efficacy of flutamide must be ascribed to a reduction of androgen synthesis and to its action on target tissues. At a dose of 250 mg/day flutamide, neither liver failure nor the side-effects generally seen with high doses (2, 4, 27, 29, 30) were observed. Moreover, a significant decrease in the cholesterol and triglycerides values was observed, in contrast to the data reported by Dodin *et al.* (4).

Finasteride, generally used at a dose of 5 mg/day (6–10), caused a significant decrease in the hirsutism score (–44%), hair diameter (–16%), and daily hair growth rate (–27%). In our experience, the results were less satisfactory than those reported by some (7–10), but in accordance with others (31, 32). The administration of finasteride has been associated with a decrease in DHT plasma values (7, 8, 10) and an increase in T levels (7–10, 31); moreover, our data showed a

decrease in DHAS levels, suggesting a slight inhibitory effect on adrenal steroidogenesis. Clinical parameters did not change, and no side-effects were reported during the administration of finasteride, confirming its favorable clinical applicability.

Using a low dose ketoconazole treatment regimen, the hirsutism score improved markedly in the patients who completed 12 months of therapy (–53%), and both hair diameter (–14%) and daily hair growth rate (–30%) decreased significantly. However, eight patients dropped out of the study because of side-effects and complications. We believe that ketoconazole should be used with caution. Our findings confirm a decrease in T, Tf, A, DHT, and DHAS values and an increase in 17-P values, and give evidence of a strong inhibition of 17-hydroxylase, 17,20-desmolase, and 11-hydroxylase activity (12, 33, 34).

High EE-CPA doses are very effective (15–18, 20); in the present study we used very low amounts of EE and CPA, and we confirm their high efficacy. Hirsutism progressively improved (as much as 60% less), and hair shaft diameter decreased significantly (–20%), even though the initial slowing of hair growth (–30% at 90 days) did not improve any further after the first 3 months of therapy. The beneficial effect of EE-CPA seems to be related to the well known peripheral effect and to the decrease in both ovarian and adrenal androgens as well as the increase in SHBG reached despite the low EE doses given. The effects of the EE-CPA on lipid metabolism are still being debated (35–38). Despite the low EE doses employed, an increase in triglycerides, which remained within the normal range, and a slight increase in total cholesterol were observed. The few transient side-effects reported during CPA-EE treatment did not require discontinuing the therapy.

In correctly evaluating and comparing the results of different treatments of hirsutism, we added the evaluation of the hair growth rate to the hirsutism score (22) and hair shaft diameter (31). This is a parameter whose behavior is partially independent of the hirsutism score and hair diameter (39), and this may explain the differences in the time response to the different therapies.

Finasteride appears to be the drug with the slowest time of action; it induces the least decrease in hirsutism score of the four treatments. However, the drug is highly effective, as the hair diameter at the end of the treatment is similar to that found with the other therapies. It is the best tolerated, and this therapy is probably the most effective in treating normoandrogenic hirsutism.

The EE-CPA combination induces the quickest reduction of hair growth and gives the greatest hair score decrease after 12 months of treatment. It is the treatment of choice in ovarian and adrenal hirsutism in sexually active women, because of steroid suppression, contraceptive effects (40) due to gonadotropin suppression, and optimal control of the menstrual cycle.

Flutamide has a quick and progressive effect on all parameters up to 12 months, very similar to the effects of EE-CPA and ketoconazole. The strong suppression of both adrenal and ovarian androgens without interference with gonadotropin secretion, the improved glucose tolerance (41), the cholesterol and triglycerides reduction, as well as the

TABLE 3. Side-effects, complications, and clinical changes during therapy with different treatment regimens

	Flutamide	Finasteride	Ketoconazole	EE-CPA
Headache	2 ^a		3	2 ^a
Nausea	1		4	
Asthenia			4	
Wt gain ^b				5
Change of libido				1
Mastodynia ^c				2
Loss of scalp hair			4	
Menstrual irregularity			3	2
AST-ALT ^c			2	
Cholesterol (mg/dL) ^d				
A	185 ± 22		180 ± 32	180 ± 38
C	165 ± 29 ^e	NS	150 ± 15 ^f	227 ± 20 ^g
Triglycerides (mg/dL) ^d				
A	63 ± 23		71 ± 18	65 ± 24
C	49 ± 12 ^e	NS	53 ± 13 ^f	106 ± 36 ^g
Satisfied		3	4	
Highly satisfied	15	10	6	20
Dissatisfied		2	6	
Drop out			8	1

Values given are the number of subjects. A, Basal; C, 360 days. Significance is given for values within the same regimen.

^a Slight and transient.

^b Mild weight gain less than 2 kg.

^c Aspartate amino-transferase-alanine amino-transferase.

^d Mean values ± DS of all treated subjects.

^e $P < 0.05$ vs. basal.

^f $P < 0.01$ vs. basal.

^g $P < 0.001$ vs. basal.

absence of side-effects at low doses enable this drug to be used in a flexible way, especially for hyperandrogenism in nonsexually active adolescents, in obese subjects, and in patients at cardiovascular risk.

Ketoconazole improves all parameters of hirsutism, as do the other treatments; however, in view of its important and frequent side-effects and complications, its use should be discouraged.

Finally, we emphasize that the treatment of hirsutism is aimed at the cause, and that each drug acts in its own way on anagen or telogen, hair diameter, or hair growth. However, the drugs employed in the present report together with spironolactone (42), which competes at the androgen receptor level, currently constitute very satisfactory alternative therapeutic regimens in the treatment of hirsutism.

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ANDROGENS

Jean D. Wilson

Androgen pharmacology is profoundly influenced by two aspects of androgen metabolism. First, testosterone, the hormone secreted by the testes, is metabolized to other hormonally active steroids in peripheral tissues; the net consequence is that the action of the hormone is the sum of the action of testosterone itself, the 5 α -reduced androgen dihydrotestosterone, and estrogenic metabolites. Testosterone and dihydrotestosterone both act via a common receptor, whereas the estrogenic metabolites act via the estrogen receptor. Pharmacological agents differ in their metabolism to these various types of active steroids. Second, testosterone and its metabolites are catabolized very rapidly by the liver. Consequently, a variety of strategies has been developed over the years to make it possible to sustain effective levels of the hormone in plasma. The most widely used means of achieving this aim is the parenteral administration of testosterone esters at 1- to 3-week intervals; these esters are hydrolyzed in vivo and release testosterone into the circulation. Transdermal delivery systems for testosterone provide an alternative to parenteral therapy and make it possible to sustain effective blood levels in the steady state. All available orally active agents to date have to either be administered very frequently or be chemically modified to slow their inactivation by the liver.

The most clear-cut indication for androgen therapy is for male hypogonadism; in such men, the restoration of plasma testosterone to normal levels replaces all known testicular functions except for spermatogenesis. Androgens also have been tried in a variety of other disorders with the hope that their pharmacological effects would provide a net benefit. For the most part, such regimens utilize orally active androgens that contain 17 α -alkyl substitutions. At present, such therapy is of clear benefit only in certain types of refractory anemia and in hereditary angioneurotic edema. Androgens also are frequently used in high doses by athletes in the belief that they enhance athletic performance.

Side effects of androgens include virilization (in women and boys), feminizing effects (men and boys), and toxic effects (all users), depending on the agent, the dose, and the duration of therapy.

Agents now are available to inhibit testosterone synthesis at the level of the pituitary (GnRH agonists; see also Chapter 55) and the testis (ketoconazole, liarazole), to prevent the conversion of testosterone to dihydrotestosterone in extraglandular tissues (finasteride), and to inhibit the binding of androgen to its receptor (flutamide, cyproterone acetate). These drugs are now undergoing evaluation in a variety of disorders in both men and women.

History. The observation that castration makes the eunuch, properly credited to primitive man, ushered in the dawn of endocrinology. The discovery that the testis is a gland of internal secretion is ascribed to Berthold, who in 1849 showed that the transplantation of gonads into castrated roosters prevents the typical signs of castration. This was the first published experimental evidence for the effect of an endocrine gland (Berthold, 1849). However, testosterone was one of the last steroid hormones to be isolated in pure form.

Chemistry. The elucidation of the chemistry of the male sex hormones was made possible by the development of methods of assay. The technique of Koch and co-workers for the determination of an-

drogenic activity utilizing the growth response of the capon's comb was used in the first isolation of the urinary principle by Butenandt (1931), who by herculean effort obtained 15 mg of androsterone (5 α -androstane-3 α -ol-17-one) from 25,000 liters of male urine.

However, androsterone could not account for the androgenic activity of testicular extracts, and the testicular hormone, testosterone, was isolated in crystalline form and identified as testosterone by David *et al.* (1935), and was then synthesized by Ruzicka and Wettstein (1935).

Testosterone is secreted by the testis and is the main androgen in the plasma of men. In women, testosterone is synthesized in small amounts by both the ovary and adrenal gland. In many target tissues

for androgens, testosterone is reduced at the 5α position to dihydrotestosterone, which serves as the intracellular mediator of most actions of the hormone. Dihydrotestosterone binds to the intracellular androgen receptor protein more tightly than does testosterone, and the dihydrotestosterone-receptor complex is more stable than the testosterone-receptor complex; its greater androgenic potency is thereby explained. A variety of additional weak androgens exist, including the testosterone precursor androstenedione, the adrenal androgen dehydroepiandrosterone, and the dihydrotestosterone metabolites 5α -androstane- $3\alpha,17\beta$ -diol and androsterone. However, these steroids bind so weakly to the androgen receptor that it is unlikely that they can act directly as hormones at physiological concentrations, and it is now believed that they are androgens only to the extent that they are converted to testosterone and/or dihydrotestosterone *in vivo*. Thus, the prior concept of weak androgens is now one of weak androgen precursors.

The major metabolites of androgens in urine—both free steroids and water-soluble conjugates—are physiologically weak or inactive; the predominant metabolites are etiocholanolone, a 5β -reduced metabolite of $\Delta^4,3$ -keto androgens, and androsterone, a metabolite of dihydrotestosterone (Figure 58-1).

Testosterone (but not dihydrotestosterone) also can be aromatized to estradiol in a variety of extraglandular tissues, a pathway that accounts for most estrogen synthesis in men and postmenopausal women (see Siiteri and MacDonald, 1973). The approximately $50\text{ }\mu\text{g}$ of estradiol synthesized each day in normal men may play a role in closure of the epiphyses; either relative or absolute excess of estrogen causes feminization in men.

Soon after the identification of testosterone as the principal testicular androgen, it became apparent that it is difficult to administer the hormone effectively by mouth or by parenteral injection. Oral administration of testosterone (or dihydrotestosterone) is followed by absorption into the portal blood and prompt degradation by the liver,

so that insignificant amounts of hormone reach the systemic circulation. Parenteral administration also is followed by prompt metabolism. It is thus necessary either to modify the androgen molecule to alter its properties, to devise means of administration that circumvent its rapid degradation, or to use a formulation that can be administered in such a way as to provide sustained physiological levels of hormone in plasma.

The aim of chemical modification is to retard the rate of catabolism or to enhance the androgenic potency of each molecule. Three general types of modification of androgens have been particularly useful. (1) Esterification of the 17β -hydroxyl group with any of several carboxylic acids decreases the polarity of the molecule, makes it more soluble in the lipid vehicles used for injection and hence slows release of the injected steroid into the circulation. The longer the carbon chain in the ester, the more lipid-soluble the steroid becomes and the more prolonged the action. Such esters are hydrolyzed before the hormone acts, and the effectiveness of drug therapy can thus be monitored by assay of plasma concentrations of testosterone. Most esters must be injected, but two such compounds, *methenolone acetate* and *testosterone undecanoate*, have features that make oral administration possible. Testosterone undecanoate is absorbed via the intestinal lymphatic ducts rather than the portal system and hence gains direct access to the systemic circulation. The methyl group on the 1 position of methenolone acetate slows hepatic inactivation and hence allows effective concentrations to be achieved in blood. (2) Alkylation at the 17α position (as in methyltestosterone and fluoxymesterone) also allows androgens to be effective orally, because the alkylated derivatives are slowly catabolized by the liver. The alkyl group itself is not removed metabolically, and hence, the alkylated derivatives mediate the action of the hormone within cells. (3) Other alterations of the structure have been made empirically. In some instances the effect is to slow the rate of inactivation; in others the modification enhances the potency; and in still others it alters the pattern of its metabolism. For example, *fluoxymesterone* is a good androgen but a poor precursor of estrogen, whereas *19-nortestosterone*, like dihydrotestosterone, binds more tightly to the androgen receptor. Most alkylated and altered steroids react poorly in the immunoassay for testosterone, and levels of these compounds in blood cannot be monitored in most clinical laboratories.

A variety of testosterone formulations has been developed to circumvent the problems inherent in rapid clearance of orally administered and parenteral agents. These include transdermal preparations (with or without emollient), pellets for subcutaneous implantation, a biodegradable microcapsule formulation for injection, and inclusion complexes that enhance sublingual absorption of the hormone. Of these, the testosterone transdermal system for use on the scrotum is the most widely tested and is now commercially available; under optimal conditions it approximates the physiological pattern of hormone levels throughout the day and provides an alternative to parenteral therapy.

Synthesis and Secretion of Testosterone. The concentration of testosterone in the plasma of males is relatively high during three periods of life: the phase of embryonic development in which male phenotypic differentiation takes place, the neonatal period, and throughout adult sexual life (see Figure 58-2). The concentration starts to rise in male embryos about the eighth week of development and declines prior to birth. It rises again during the neonatal

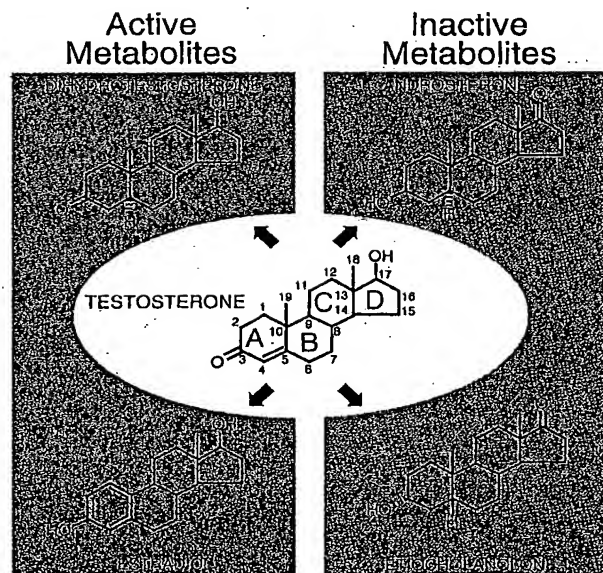


Figure 58-1. Metabolism of androgens.

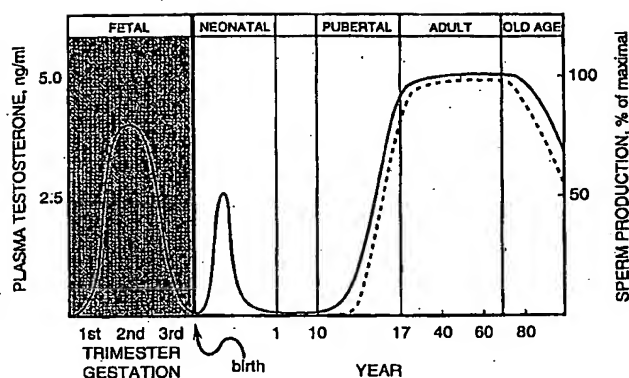


Figure 58-2. Schematic diagram of phases of male sexual function as indicated by mean plasma testosterone level (solid line) and sperm production (dashed line) at different phases of life.

(From Griffin and Wilson, 1980, with permission.)

tal period and then falls to typical prepubertal values within the first year of life. At the time of male puberty, the pituitary begins to secrete increased amounts of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Gonadotropins are initially secreted in a cyclic fashion in synchrony with the sleep cycle. As puberty progresses, however, pulsatile secretion of gonadotropins occurs during both sleep and waking periods (see Boyar, 1978). The hypothalamus and pituitary become less sensitive to feedback inhibition by sex hormones during puberty. The initiating event for these phenomena is unknown.

Prior to puberty, concentrations of testosterone in plasma are low (less than 20 ng/dl [0.7 nM]), although the immature testes are capable of synthesizing androgens if challenged with gonadotropin. In the adult male, plasma testosterone concentrations are between 300 and 1000 ng/dl (10 to 35 nM), and the rate of production is 2.5 to 11 mg per day (Rosenfield, 1972). Pathways of androgen biosynthesis are shown in Figure 57-1. In plasma, about 40% of testosterone is bound to sex hormone-binding globulin and about 2% is free (unbound); the remainder is bound to albumin and other proteins. Albumin-bound testosterone can dissociate within a capillary bed, such that the fraction available for rapid entry into cells is actually about half of the total (see Partridge, 1986).

Androgen-Gonadotropin Relationships. Gonadotropins and testosterone are secreted in a pulsatile manner. In adult men, the concentrations of LH, FSH, and testosterone in plasma fluctuate during the course of the day, although integrated daily values are relatively constant.

LH and FSH together regulate testicular growth, spermatogenesis, and steroidogenesis. Growth hormone may have a synergistic effect with LH on the testis, while estrogens can decrease the effects of LH on the secretion of testosterone. The actions of the gonadotropins are mediated at least in part through cyclic AMP (see Cooke *et al.*, 1992). LH interacts with the interstitial (Leydig) cells of the testes to increase the synthesis of cyclic AMP and subsequently the conversion of cholesterol to androgens. Cyclic AMP enhances the activity of several enzymes in the steroidogenic pathway, including the cholesterol side chain cleavage enzyme, and it also may influence the availability of cholesterol to serve as substrate (see Miller, 1988). The major action of FSH is to promote spermatogenesis in the seminiferous tubules, and that of LH is to regulate testosterone synthesis by Leydig cells. FSH also can augment the activity of LH and enhance testosterone synthesis (see Lipsett, 1980). Furthermore, testosterone is required for spermatogenesis and maturation of sperm. With immunohistochemical techniques, LH is localized principally in Leydig and peritubular cells, and FSH binds to Sertoli cell in the spermatogenic tubule (Castro *et al.*, 1972; Ritzén *et al.*, 1989). Androgens released from the Leydig cell diffuse into the spermatogenic tubule to promote spermatogenesis and gain access to the circulation to virilize the male at puberty (Matsumoto, 1989). Both LH and FSH have growth promoting effects on the testes. In the human testis the effects of human chorionic gonadotropin appear to be identical with those of LH.

Administration of testosterone to intact animals suppresses the secretion of LH and thereby causes atrophy of interstitial tissue. The administration of testosterone also suppresses the excessive secretion of FSH in eunuchism, but whether testosterone plays a major role in the physiological regulation of FSH secretion is not resolved. Implantation of testosterone in the median eminence of rats inhibits pituitary gonadotropin secretion by decreasing the concentration of gonadotropin-releasing hormone (GnRH; formerly referred to as luteinizing hormone-releasing hormone, LHRH) (see Schally, 1978; see also Chapter 55). Likewise, administration of testosterone to hypogonadal men causes a decrease in both the frequency and amplitude of pulses of LH secretion, presumably by inhibiting the release of GnRH (Matsumoto and Bremner, 1984).

In normal men, about 15% of the estradiol is secreted from the testes, probably the Leydig cells (see Lipsett, 1980). Estrogens in men also are synthesized from androgens in extraglandular tissues, including the brain (see Siiteri and MacDonald, 1973; Marcus and Korenman, 1976). Estrogens formed locally in the brain from admin-

istered or endogenous androgen may play a role in the regulation of gonadotropin secretion by testosterone.

Feedback inhibition of FSH secretion by testicular hormones involves peptides as well as steroids (Keogh *et al.*, 1976; Ramasharma and Sairam, 1982). Inhibin is a peptide hormone that contains both 20,000- and 15,000-dalton subunits (*see* Baird and Smith, 1993). The protein is synthesized by both Sertoli cells and the ovary and is believed to decrease production of the mRNA for the β subunit of FSH in the pituitary (Findlay *et al.*, 1991).

Ovarian and Adrenal Androgens. Testosterone and other androgens are secreted by the ovary and the adrenal cortex as well as by the testis. Furthermore, androstenedione and dehydroepiandrosterone, which are also produced by both the ovary and adrenal, can be converted to testosterone and estrogen in peripheral tissues. The daily rate of production of testosterone in women is about 0.25 mg, and about one-half of this is derived from the metabolic conversion of androstenedione to testosterone at extraglandular sites (*see* Rosenfield, 1972; Givens, 1978). The synthesis and secretion of testosterone in rabbit ovary are enhanced by the administration of LH (Hilliard *et al.*, 1974).

Alterations in plasma concentrations of testosterone and androstenedione occur during the menstrual cycle. The concentration of testosterone in the plasma of women ranges from 15 to 65 ng/dl (0.5 to 2.3 nM). Two peaks of androgen concentration correspond to those of plasma estrogens at the preovulatory and luteal phases of the cycle (Judd and Yen, 1973). In some ovarian disorders, androgens are secreted in increased amounts by the ovary, resulting in virilization.

In normal men, production of testosterone by the adrenal cortex is not sufficient to maintain spermatogenesis or secondary sexual features in the adult. However, in certain conditions such as congenital adrenal hyperplasia and adrenal tumors, the adrenal cortex can secrete large quantities of androstenedione, and significant amounts of testosterone can be formed from this precursor in extraglandular sites.

Physiological and Pharmacological Actions. Androgens serve different functions at different stages of life. During embryonic life, they virilize the urogenital tract of the male embryo, and their action is thus essential for the development of the male phenotype. The role of androgens, if any, during the neonatal surge of androgen secretion is not defined but may involve developmental functions within the central nervous system. At puberty, the hormones act to transform the boy into a man. Minimal androgen secretion from the prepubertal testis and adrenal

cortex suppresses secretion of gonadotropins until, at a variable age, secretion of gonadotropins becomes less sensitive to feedback inhibition and the testes start to enlarge (*see* Franchimont, 1977; Boyar, 1978). Shortly thereafter, the penis and scrotum begin to grow, and pubic hair appears. Early in puberty penile erections, nocturnal ejaculations, and masturbation become frequent in most boys. Almost simultaneously the growth-promoting property of androgen causes an increase in height and the development of the skeletal musculature, which contribute to a rapid increase in body weight. As the muscles grow, physical vigor is increased. The testes reach adult proportions before all the changes of puberty are completed. As a result of the actions of androgens, the skin becomes thicker and tends to be oily owing to a proliferation of sebaceous glands; the latter are prone to plugging and infection, predisposing to acne. Subcutaneous fat is lost, and the veins are prominent under the skin. Axillary hair grows, and hair on the trunk and limbs develops into a pattern typical of the male. Growth of the larynx causes difficulty at first in adjusting the tone of speech and later brings about a permanent deepening of the voice. Growth of beard and body hair lags behind the other events of puberty and is the last of the secondary sex characteristics to develop. Concurrently, those whose inheritance so dictates show the first signs of male pattern baldness, with recession of the hairline at the temples and thinning of the hair at the crown. At about this time, the major spurt in growth comes to an end as the epiphyses of the larger long bones begin to close, and over the next few years only 1 to 2 cm of additional growth is usual.

Androgens also may be responsible in part for the aggressive and sexual behavior of males (*see* Lunde and Hamburg, 1972; Wilson, 1982) and, in some species, for organizational effects in the brain during prenatal or early postnatal life (*see* Pardridge *et al.*, 1982). While this is a difficult matter to resolve, the differential behavior patterns of many male and female animals suggest that sex hormones play an important role in behavior. Although psychopathic behavior in men is not associated with altered patterns of androgen metabolism, administration of pharmacological amounts of androgen to normal men for short periods can cause a variety of adverse effects on mood and behavior (Su *et al.*, 1993).

When androgen is given before puberty or to a young eunuchoid man, the events of normal puberty are duplicated, and the time required to complete normal pubertal virilization (2 or more years) is not significantly shortened. Shortly after the start of treatment, erections appear and may be embarrassingly inappropriate and frequent, even to the point of discomfort; with continued treatment at the

same dose this response subsides. Increased physical vigor is noted within a few weeks, and a general feeling of well-being ensues. A distinct change in the voice can be noted; soon thereafter the penis begins to grow, and axillary and pubic hair become more luxuriant. The rapidity of skeletal growth is impressive in boys treated at or before the time of normal puberty; the height may increase 10 cm or more during the first year and continue at a diminished rate for 2 or 3 years. With continued treatment, development follows the course of normal puberty, with the growth of a beard and body hair as a late expression of therapy.

Failure of Puberty Owing to Hypogonadism. The normal actions of androgens are illustrated by the consequences of deficiency. If the testes fail to function or are removed in boyhood, puberty does not occur. Failure of the testes to develop may result from a deficiency of gonadotropins or from a primary testicular defect. A boy so afflicted continues to grow and becomes abnormally tall; the hands and feet become especially large, and the limbs are unduly long. The childish appearance and demeanor are in striking contrast to the stature; the larynx does not grow, leaving the voice high-pitched. The skeletal musculature is underdeveloped, and bone density is subnormal. Accumulation of fat is especially prominent around the shoulders and breasts and over the upper thighs, hips, and abdomen, the whole giving the mistaken impression of femininity. Male-pattern baldness does not appear, the beard is scant or nonexistent, the axillary and pubic hair is sparse, and the body hair is short and fine. The genitalia are those of a child, and there is no sex drive.

Hypogonadism after Puberty. Some of the sex characteristics developed during puberty are self-sustaining, while others require the continued action of androgen. Hypogonadism in the adult is typified by castration after puberty. The general bodily proportions remain the same, the penis does not shrink, the voice does not change, and the beard and body hair remain unchanged for a long time. Libido is greatly reduced or absent; erections during sleep are decreased, but erections are normal after erotic visual stimuli (Carani *et al.*, 1992). The prostate and seminal vesicles regress and the volume of the semen is small or there is none at all. Osteopenia is usual.

Complete failure of the endocrine function of the testis in adult life is not common; a partial deficiency can originate from incomplete testicular development at puberty, as in 47,XXY men with the Klinefelter syndrome, or from a disorder during adult life, such as pituitary failure or a viral infection of the testis (see Odell and Swerdloff, 1978). Testicular function decreases gradually with age, beginning in the fifth decade. However, the decrease in erectile function that often occurs in aging men usually cannot be attributed to altered testicular function (Gray *et al.*, 1991).

Actions on the Testis and Accessory Structures. At about the eighth week of fetal life, testicular androgens begin to be secreted and express their important role in the differentiation and development of the male reproductive tract (see Jost, 1971). Lack of androgens in the male fetus results in the development of a female external phenotype. The developing testes also produce a peptide hormone (Müllerian inhibiting substance) that causes regression of the Müllerian ducts of the fetus (see Donahoe *et al.*, 1987).

Subsequently, under the influence of testosterone, the Wolffian ducts differentiate into the epididymis, vas deferens, and seminal vesicles. Dihydrotestosterone causes fusion and elongation of the labioscrotal fold to result in the development of the male urethra, penis, and scrotum, and virilization of the urogenital sinus to form the prostate. During the latter part of gestation, plasma concentrations of androgen in the male fetus begin to decline, and at birth they are essentially undetectable (see Rosenfield, 1972).

At puberty and thereafter androgens exert a direct effect upon the testis. Androgens are required for spermatogenesis in the seminiferous tubules and for the maturation of sperm in passage through the epididymis and vas deferens. These processes are ordered and complex, and the nature of the effects of testosterone thereon is unknown. Studies of these events are complicated by the fact that 10 weeks are required for completion of spermatogenesis and that 2 to 3 additional weeks are needed for passage of sperm through the vas deferens and for maturation of sperm.

In fetal, prepubertal, and pubertal life the actions of testosterone result in growth of the clitoris or penis. Androgens also control the growth and function of the seminal vesicle and prostate.

Anabolic Effects. The nitrogen-retaining effect of androgen was first demonstrated in castrated dogs (Kochakian and Murlin, 1935). Papanicolaou and Falk (1938) showed that certain skeletal muscles of male guinea pigs are larger than those of the female and that the difference is abolished by removal of the testes. Administration of testosterone to the female or the castrated male causes male muscle development; thus, male muscle development is a phenotypic characteristic dependent upon androgen for its expression. In human beings, the major difference in muscle development between the sexes is in the muscles of the shoulder girdle. The anabolic actions of androgens are mediated by the same receptor protein that mediates the actions of the hormone in other target tissues (see below; see also Saartok *et al.*, 1984).

The anabolic (nitrogen-retaining) effects of androgen are more pronounced in hypogonadal men, in boys before puberty, and in women than in normal men (Knowlton *et al.*, 1942). Indeed, normal men experience only a transient positive nitrogen balance of a slight degree when usual pharmacological amounts of androgens are administered. A dose of 25 mg of testosterone propionate daily causes an average daily retention of nitrogen of 63 mg/kg of body weight in hypogonadal men. There also is retention of K^+ , Na^+ , Cl^- , phosphate, and sulfur and a gain in weight, which can be accounted for largely by the water held in association with the retained electrolytes and protein. When the administration of androgen is stopped, Na^+ , Cl^- , and water quickly are lost from the body, phosphate and K^+ are lost less rapidly and completely, and the stored nitrogen is retained for weeks.

Effects on Sebaceous Glands. Development of acne at puberty is related to the growth and secretion of the sebaceous glands. The administration of exogenous androgens also may cause acne, but this effect is usually less pronounced in adult men (see Ebling, 1970).

Mechanism of Action. At many sites of action, testosterone is not the active form of the hormone. It is converted by steroid 5α -reductases in target tissues to the more active dihydrotestosterone (Table 58-1; see Russell and Wilson, 1994). Steroid 5α -reductase 1 is located largely in nongenital skin and liver, and steroid 5α -reductase 2 is present principally in the urogenital tract of the male and in the genital skin of both sexes. In one form of male pseudohermaphroditism, the target tissues are deficient in steroid 5α -reductase 2. In this disorder, the genotypic male secretes normal amounts of testosterone from the testes, but the hormone is not converted to dihydrotestosterone, and male external genitalia fail to develop. In contrast, virilization of the Wolffian ducts during embryogenesis is normal in such individuals; testosterone itself also is believed to be the principal mediator of the regulation of LH production by the hypothalamic-pituitary system and of spermatogenesis (Griffin *et al.*, 1994).

Testosterone or dihydrotestosterone binds to an intracellular protein receptor (Figure 58-3), and the hormone-receptor complex is attached in the nucleus to specific hormone regulatory elements on the chromosomes and acts to increase the synthesis of specific RNAs and proteins. The human androgen receptor is a typical member of the superfamily of steroid and thyroid hormone receptors (see Carson-Jurica *et al.*, 1990; see also Chapter 2). It is encoded by a gene on the X chromosome and contains androgen-binding, DNA-binding, and functional domains (Chang *et al.*, 1988). The general mechanism by which testosterone and dihydrotestosterone are believed to act to

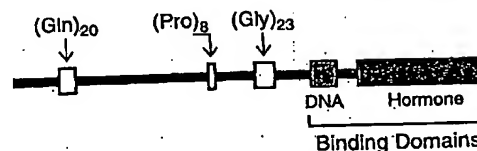


Figure 58-3. Schematic diagram of the human androgen receptor, which contains 917 amino acids.

In the N-terminal domain, there are three homopolymeric repeat regions, one with a modal number of 20 glutamines (Gln), one with 8 prolines (Pro), and one with 23 glycines (Gly).

promote virilization of the male is illustrated schematically in Figure 58-4. Mutations that impair either the function of the 5α -reductase or the androgen receptor impair the virilization of the male embryo and result in male pseudohermaphroditism (see Griffin *et al.*, 1994).

Absorption, Metabolism, and Excretion. Testosterone injected as a solution in oil is quickly absorbed, metabolized, and excreted so that the androgenic effect is small. Testosterone given by mouth is readily absorbed but is even less effective, since most of the hormone is absorbed into the portal circulation and metabolized by the liver before reaching the systemic circulation. Alternate means of administering testosterone have been devised to circumvent these difficulties (see above).

Testosterone esters are less polar than the free steroid and, when such esters are injected intramuscularly in oil, are absorbed more slowly. For example, testosterone propionate is more active than testosterone, even when each is injected every day. The cypionate and enanthate esters are fully effective when given at 1- to 3-week intervals in proportionately larger doses, and the ultra-long-acting ester testosterone buciclate can be administered at 12 week intervals

Table 58-1
Comparison of Human Steroid 5α -Reductases 1 and 2

	5α -REDUCTASE 1	5α -REDUCTASE 2
pH optimum	7.5	5.0
Location of gene	Chromosome 5	Chromosome 2
Tissue distribution	Liver and non-genital skin	Liver and male urogenital tract
K_m for testosterone	4 μ M	1 μ M
K_i for finasteride	300 nM	3-5 nM
Activity in steroid 5α -reductase deficiency	Normal	Low or absent
Sequence homology	Approximately 50%	

SOURCE: Adapted from Russell and Wilson, 1994, with permission.

(Behre and Nieschlag, 1992). Since these esters are hydrolyzed prior to action, the concentrations of testosterone in plasma can be monitored by conventional immunoassay. This greatly facilitates the administration of effective dosages for each patient (Caminos-Torres *et al.*, 1977).

Testosterone is inactivated primarily in the liver. Metabolism to androstenedione involves oxidation of the 17-OH group; 5 α -reduction of ring A of androstenedione leads to formation of androstenedione, and the 3-keto group is reduced to form androsterone; alternatively, androstenedione can be reduced in the 5 β position and can undergo 3-keto reduction to form etiocholanolone (see Figure 58-1). Dihydrotestosterone itself is converted in the liver to androsterone, androstenedione, and androstenediol (see Fotherby and James, 1972). Alkylation of androgens at the 17 position markedly retards their hepatic metabolism and permits such analogs to be effective orally (see below).

After the administration of radiolabeled testosterone, about 90% of the radioactivity appears in the urine; 6% appears in the feces after undergoing enterohepatic circulation. Urinary products include androsterone and etiocholanolone. Small amounts of androstenediol and estrogens also are excreted, largely as glucuronide and sulfate conjugates.

Androsterone and etiocholanolone, among many other compounds, are measured as urinary 17-ketosteroids in the usual clinical tests. However, the major fraction of the ketosteroids of urine consists of metabolic products of the adrenal steroids. Thus, measurement of the excretion of 17-ketosteroids is not a valid test for the functional activity of the testis. Low values may point to adrenal insufficiency rather than to hypogonadism, and high values almost always indicate adrenocortical hyperactivity or tumor. When the testes are absent, the human male is androgen-deficient even though the urinary 17-ketosteroids may be within the normal range. Likewise, in women, measurement of the excretion of 17-ketosteroids rarely is

helpful in elucidating whether an excess of androgen originates in the ovary or the adrenal.

The esters of testosterone are hydrolyzed to free testosterone and subsequently are metabolized in the same way as is testosterone itself, but many other changes in the molecule (as in methyltestosterone and fluoxymesterone) alter the course of metabolic degradation. As a result, many synthetic androgens are metabolized less rapidly than is testosterone and have longer half-lives. Unaltered compounds, metabolites, and conjugates are excreted in the urine and feces (Fotherby and James, 1972). The fact that these altered steroid molecules and their metabolites are excreted in urine allows their use to be detected when they are taken for performance enhancement by athletes (see Wilson, 1988).

Assays. Bioassay is used in the evaluation of androgenic potency of new compounds. The classical assay is based on the growth of the comb of the capon; the most widely used test depends upon the growth of the seminal vesicles or ventral prostate of the castrated rat. The search for anabolic steroids made use of a different series of bioassays, including assessment of the growth of the kidney or levator ani muscle of castrated rats. Unfortunately, none of these assays is totally satisfactory and able to predict the results obtained in clinical trials, and no pure anabolic steroid without androgenic effects has ever been described. The failure to separate the androgenic and anabolic effects is not surprising, since androgenic and anabolic actions of the hormone are mediated by a single receptor protein.

Administration and Dosage. Some of the parenteral and oral preparations of androgens available for clinical use are summarized in Tables 58-2 and 58-3, respectively.

Androgen therapy is used primarily in androgen-deficient men for the development or maintenance of secondary sex characteristics. When replacement therapy with androgen is required, the intramuscular preparations are the most effective. Dosage should provide

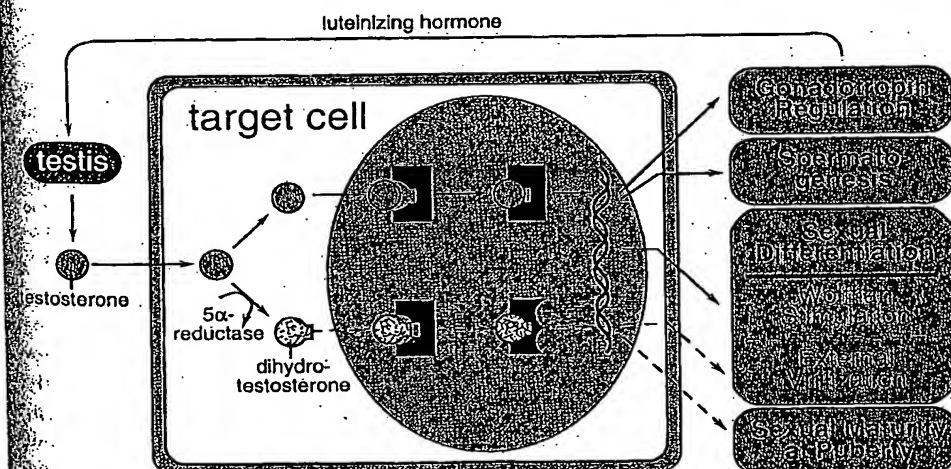
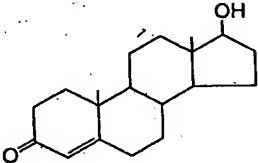
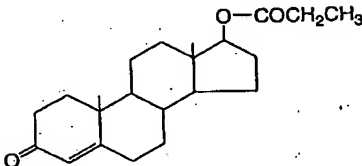
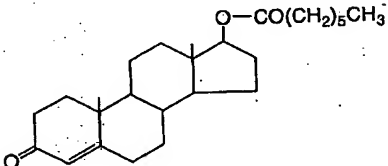
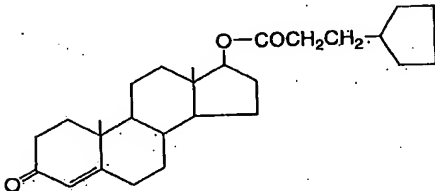


Figure 58-4. A schematic diagram of the mechanisms by which androgen acts in target tissues to perform its major functions. (Reprinted, by permission of the *New England Journal of Medicine*, 326:611, 1992.)

Table 58-2
Some Parenteral Androgens Used in Therapy

NONPROPRIETARY NAME AND SOME TRADE NAMES	CHEMICAL STRUCTURE	DOSAGE FORMS AND USUAL DOSAGE FOR ANDROGEN DEFICIENCY
Testosterone TESTOJECT-50		Aqueous suspension for intramuscular use; 10 to 50 mg three times weekly
Testosterone propionate TESTEX		Oily solution for intramuscular use: 10 to 25 mg two to three times weekly
Testosterone enanthate DELATESTRYL		Oily solution for intramuscular use: 50 to 400 mg every 2 to 4 weeks
Testosterone cypionate DEPO-TESTOSTERONE		Oily solution for intramuscular use: 50 to 400 mg every 2 to 4 weeks

about 6 to 10 mg per day; with testosterone propionate, this is met by giving 25 mg three times weekly. With the longer-acting esters, the dose is about 200 mg every 2 to 3 weeks. Long-term treatment with these doses ordinarily causes full masculine development, provided therapy is started sufficiently early in life. When androgen replacement is started late (over the age of 25) the degree of virilization attained is variable but may be near normal.

A preparation of testosterone for transdermal use has been developed in which a testosterone-loaded film is applied each day to the scrotal skin (TESTODERM; testosterone transdermal system). This preparation permits maintenance of plasma concentrations of testosterone within the normal range, while circumventing the necessity for parenteral administration (Findley *et al.*, 1987; Bals-Pratsch *et al.*, 1988). Such therapy causes a disproportionate increase in the plasma concentration of dihydrotestosterone to a level that is 30% to 40% of that of testosterone, presumably because of conversion by the scrotal skin during absorption; however, dihydrotestosterone has no known deleterious effects at these concentrations. A transdermal preparation that does not require applica-

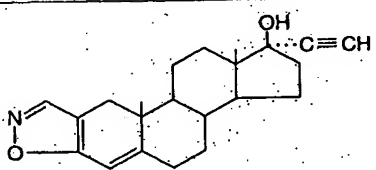
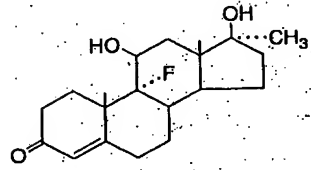
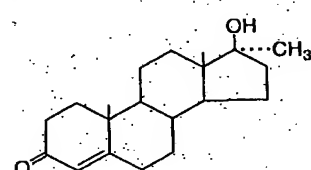
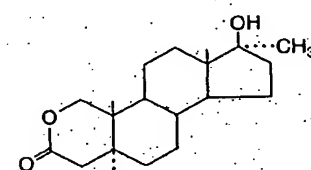
tion to the scrotal skin also has been developed (Meikle *et al.*, 1992).

Some preparations of androgens have been introduced primarily for use as anabolic agents, with the expectation that they would be relatively less androgenic than testosterone and its close relatives. However, none is free of androgenic activity.

Various mixtures of androgenic and anabolic steroids with estrogens, vitamins, and other agents also are available. However, the use of these fixed-dose combinations is to be discouraged. In particular, their prolonged use in postmenopausal women and geriatric patients is costly and usually irrational.

Untoward Effects. Three types of side effects of androgens can be recognized: (1) virilizing side effects are mediated by the androgen receptor and are inappropriate only when the recipient is not a hypogonadal adult male; (2) feminizing side effects are mediated by estrogenic metabolites of the administered steroid; and (3) toxic side

Table 58-3
Some Oral and Buccal Androgens Used in Therapy

NONPROPRIETARY NAME AND SOME TRADE NAMES	CHEMICAL STRUCTURE	DOSAGE FORMS AND USUAL DOSAGE
Danazol* DANOCRINE		Capsules: 200 to 800 mg daily
Fluoxymesterone HALOTESTIN		Tablets: 2.5 to 20 mg daily
Methyltestosterone ORETON METHYL, TESTRED, VIRILON		Tablets and capsules: 10 to 50 mg daily Buccal tablets: 5 to 25 mg daily
Oxandrolone† OXANDRIN		Tablets: 2.5 to 20 mg daily

*Used predominantly to suppress the pituitary and for the treatment of hereditary angioneurotic edema.

†Investigational; orphan drug status.

effects are mediated generally by uncertain mechanisms. **Virilizing Effects.** When used in women, all androgens carry the risk of causing masculinization. Among the undesirable manifestations are acne, the growth of facial hair, and coarsening of the voice. Menstrual irregularities occur if gonadotropin secretion is suppressed. If treatment is discontinued as soon as the initial symptoms are noticed, they slowly subside. With continued treatment—as in the long-term use of androgen in mammary carcinoma—male-pattern baldness, excessive body hair, prominent musculature, and hypertrophy of the clitoris also may develop. With prolonged treatment many of these effects, such as the deepening of the voice, are irreversible. During initial androgen-replacement therapy in hypogonadal males, sustained erections may be seen. This effect subsides with continued therapy at the same or lower doses of androgen.

Profound virilization and serious disturbances of

growth and osseous development can occur when androgens are given to children. The capacity of androgens to enhance epiphyseal closure in children may persist for as long as several months after discontinuation of the drug. Androgens should be used with great care in children and should not be used during pregnancy, since they can cross the placenta and masculinize the female fetus.

Although androgens are required for spermatogenesis and may maintain spermatogenesis for prolonged periods in animals after hypophysectomy, continued use of androgens in normal men may result in azoospermia owing to inhibition of gonadotropin secretion and conversion of androgens to estrogens. Anabolic steroids may produce the same effect, and diminution in sperm count can sometimes persist for many months after the administration of anabolic steroids is stopped.

Feminizing Effects. Feminizing side effects, particularly

gynecomastia, can occur in men who receive androgens. As stated above, androgens containing a $\Delta^4,3$ -keto configuration can be converted (aromatized) to estrogens in extraglandular tissues, and the administration of testosterone esters causes an increase in plasma concentrations of estrogen. The feminizing side effects are particularly severe in children (possibly because of an increased extraglandular aromatase activity compared with adults) and in men with liver disease (who have diminished rates of androgen clearance and hence shunt androgen substrate to extraglandular sites of aromatization). Feminization is not a side effect of androgens such as 19-nortestosterone and fluoxymesterone which are poor substrates for the aromatase enzyme.

Toxic Effects. Edema. Retention of water in association with sodium chloride appears to be a consistent effect of the administration of androgen and accounts for much of the gain in weight, at least in short-term treatment. In the doses used to treat hypogonadism, retention of fluid usually does not lead to detectable edema, but edema may become troublesome when large doses are given in the treatment of neoplastic diseases. Edema also is common in patients with congestive heart failure or renal insufficiency and in patients prone to edema from some other cause, such as cirrhosis of the liver or hypoproteinemia. Salt and water retention from androgens usually responds to the administration of natriuretics.

Jaundice. Methyltestosterone was the first androgen discovered to cause cholestatic hepatitis, but all androgens with 17 α -alkyl substitutions can cause this complication. Jaundice is the prominent clinical feature, and the underlying disturbance is stasis and accumulation of bile in the biliary capillaries of the central portion of the hepatic lobules, without obstruction in the larger ducts (see Ishak, 1981). The hepatic cells usually exhibit only minor histological changes and remain viable. If jaundice occurs, it generally develops after 2 to 5 months of therapy. Alterations in various tests of hepatic function occur more commonly than jaundice and include increases in the concentration of bilirubin and the levels of aspartate aminotransferase and alkaline phosphatase in the plasma. The severity of the response is dependent on the dose of 17 α -alkyl substituted testosterone analogs administered and is particularly prominent when large amounts are given, as for palliation in neoplastic diseases. Disturbance of hepatic function has not been described with the parenteral use of testosterone esters. Consequently, testosterone esters should be administered instead of 17 α -substituted steroids in virtually all clinical situations (except hereditary angioneurotic edema). In particular, the use of 17 α -substituted esters should be avoided in patients with liver disease. Other forms of hepatic disease, such as peliosis hepatitis, also are rarely as-

sociated with the use of androgens (see Ishak, 1981).

Hepatic Carcinoma. Patients who have received 17 α -alkyl substituted androgens for prolonged periods may develop hepatic adenocarcinoma. Most of the patients described received the derivatives for 1 to 7 years, and the complication may be more common in subjects with Fanconi's anemia (see Ishak, 1979).

Effects on Laboratory Tests. Androgens can decrease the concentration of thyroid-binding globulin in plasma and thereby influence thyroid function tests, increase the excretion of 17-ketosteroids, raise plasma LDL-cholesterol and lower plasma HDL-cholesterol concentrations, and increase the hematocrit (see also Chapters 36 and 54). 17 α -Alkyl-substituted steroids cause an increase in the hepatic synthesis and plasma concentrations of a variety of glycoproteins (Barbosa *et al.*, 1971). Alterations in tests of hepatic function are discussed above.

Therapeutic Uses. The clearest therapeutic indication for androgens is deficient endocrine function of the testes. In addition, they have been tried in a variety of other situations in the hope that their effects on nongenital tissues would be beneficial. Testosterone esters are the preferred agents in all situations. Because of their toxic side effects (see above), the use of alkylated androgens should be restricted to hereditary angioneurotic edema (see below) or short-term therapy in patients with serious illnesses.

Hypogonadism. Failure of the testes to secrete androgen usually is first evident when the changes of puberty seem to be delayed. The age of onset of puberty varies widely among individuals, and when no evidence of maturation is seen by age 15 to 17, there may be great concern on the part of the patient and his parents. There is debate about the use of androgens to hasten the changes of puberty in normal boys with delayed sexual maturation. Most physicians would agree that androgens should be withheld unless the psychological well-being is jeopardized.

Patients with delayed puberty should be evaluated for pituitary as well as gonadal dysfunction. Hypogonadism may be due to primary testicular failure or to diminished concentrations of gonadotropins. The latter can result from hypopituitarism, as discussed below, or from low concentrations of GnRH. The uses of gonadotropins and GnRH for secondary hypogonadism are discussed in Chapter 55.

If an androgen is administered to boys with delayed puberty in the absence of an established diagnosis of hypogonadism, it can be given in courses of 4 to 6 months at a time and stopped for like periods to ascertain whether the testes will enlarge and development will progress spontaneously. The secretion of gonadotropins also must be reevaluated after discontinuation of androgens. Such regimens do not have an adverse effect on final height (Uruena *et al.*, 1992).

When testicular failure is complete and puberty cannot occur, prolonged therapy is required. One of the long-acting esters of testosterone, such as the cypionate or the enanthate, may be given intramuscularly. It is recommended that initial doses of about one-half of the eventual maintenance dose be given for 6 months to 1 year; the

eventual maintenance dose of the long-acting esters of testosterone is about 200 mg every 2 weeks. The concentration of testosterone in plasma should be titrated to the normal range in all individuals (Caminos-Torres *et al.*, 1977). As emphasized above, because of their effects on hepatic function, 17 α -alkyl-substituted androgens should not be used for replacement therapy.

When therapy is begun at the time of expected puberty in boys with either primary or secondary hypogonadism, the normal events of puberty proceed in the usual fashion. The normal growth spurt occurs, and penile development, deepening of the voice, and appearance of other secondary sex characteristics are apparent during the first year. Puberty in normal boys extends over several years, and treatment designed to replicate normal development cannot hasten the process greatly. Testosterone exerts its full action only in the presence of a balanced hormonal environment and specifically only in the presence of adequate concentrations of growth hormone. Consequently, prepubertal boys with coexisting deficiency of growth hormone exhibit a diminished response to androgens with regard to both growth and virilization unless growth hormone is given simultaneously.

If therapy is delayed until long after the usual time of puberty, the degree of virilization that can be achieved is variable. Many of these patients undergo a late but relatively complete anatomical and functional male maturation. If hypogonadism is primary and of long duration, suppression of plasma concentrations of LH to the normal range may not occur for months. In subjects with hypogonadotropic hypogonadism, androgen therapy is given until fertility is desired, at which time gonadotropin or GnRH is administered to promote spermatogenesis (see Chapter 55). As would be expected, androgen replacement in hypogonadal men causes the prostate and seminal vesicles to increase to the normal adult male range (Sasagawa *et al.*, 1990).

In postpubertal testicular failure, even of many years duration, resumption of normal sexual activity usually occurs following adequate replacement. The major effect of androgen on sex drive appears to be on libido; the volume of the ejaculate and other secondary sex characteristics return to normal, and the effects of pubertal androgen on hemoglobin, nitrogen retention, and skeletal development also are reproduced. In contrast, administration of testosterone has no effect on libido in men with normal concentrations of the hormone in plasma.

Nitrogen Balance and Muscle Development. In hypogonadal or castrated men, androgens cause reduction in the urinary excretion of nitrogen, Na⁺, K⁺, and Cl⁻ and a gain in weight (see Wilson and Griffin, 1980). In all situations other than hypogonadism, the positive nitrogen balance is short lived (probably lasting no more than 1 to 2 months).

Since androgens have significant effects on muscle mass and on body weight when administered to hypogonadal men, it was assumed, but never proven, that androgens in pharmacological doses could promote growth of muscle above the levels produced by the normal testicular secretion. This assumption was based on the belief that anabolic and androgenic actions are different, and a concerted effort was made to devise pure "anabolic" steroids that have no androgenic effects. In fact, androgenic and anabolic effects do not result from different actions of the same hormone but represent the same action in different tissues; androgen-responsive muscle contains the same receptor that mediates the action of the hormone in other target tissues (Saartok *et al.*, 1984). All anabolic hormones tested to date are also androgenic. In appropriate doses, most anabolic agents can be used for replacement of androgen. For example, *methandrostenolone*,

which has a greater effect on nitrogen balance per unit weight than does methyltestosterone, is a potent androgen and has been used for replacement therapy in hypogonadal men. Nevertheless, androgens have been tried in a variety of clinical situations other than hypogonadism with the hope that improvement in nitrogen balance and muscle development would outweigh any deleterious side effects.

Catabolic States. Body protein is broken down more rapidly than it is formed following injury or surgery, and excess nitrogen is excreted in the urine as a consequence. During the subsequent recovery phase, nitrogen deficits are replaced. Anabolic steroids can improve the nitrogen balance during the first few days following relatively minor operations in well-nourished subjects, but the decrease in nitrogen loss is minimal and has not been shown to be of significant therapeutic benefit. Likewise, effects of androgens on weight in undernourished, debilitated, or elderly individuals are due predominantly to enhancement of appetite. In appropriately controlled studies, no consistent effects on weight or strength have been documented following treatment with androgen. These negative results are probably the consequence of several factors, including the dependence of anabolic effects on adequate nutrition and health, the paucity of effects of androgens in men with normal concentrations of testosterone, and the temporary nature of any positive nitrogen balance when it does occur. In short, androgens are ineffective in promoting anabolism in acute illness, severe trauma, and protein depletion associated with chronic illness (see Wilson and Griffin, 1980). Androgens also are of little value in the management of nitrogen accumulation in chronic renal failure; at best they induce a transient improvement in nitrogen balance that is of doubtful importance. In acute renal failure, androgens cause a decrease in the rate of production of urea and a consequent decrease in the frequency of dialysis required for some patients. Most patients in this clinical setting do well without androgen therapy.

Athletic Performance. Androgens sometimes are used by athletes in the belief that athletic performance will be improved. Weight lifters and body builders began to use the drugs in the 1950s, and androgen abuse subsequently became widespread at all levels of athletic competition. Indeed, the problem continues to grow in magnitude and has received a great deal of attention in the press as a consequence of the disqualification of athletes at competitions. Many athletes who abuse androgens, including those who use veterinary drugs not approved for human use, obtain them through the "steroid underground"; others obtain them through physicians' prescriptions. The fact that any abusers obtain androgens from physicians is particularly worrisome, because many aspects of androgen abuse are poorly understood.

Androgens do promote muscle growth in boys and in women of all ages, and this phenomenon is mediated by the androgen receptor. However, it is not known whether androgens have any beneficial effects on muscle development, nitrogen balance, or athletic performance in sexually mature men. Appropriately controlled studies of the effects of androgens on strength and performance in conditioned athletes have yielded inconclusive results. If androgens do have a beneficial effect, it is not known how they work, since the androgen receptors in mature men appear to be functionally saturated. Two pieces of evidence do suggest that massive doses of androgen may enhance muscle development in men. Namely, administration of pharmacological amounts of testosterone enanthate causes an increase in lean body mass (Forbes *et al.*, 1992) and an increase in whole-body protein synthesis (Griggs *et al.*, 1989). High doses of androgens may act at the level of the glucocorticoid receptor to inhibit the catabolic effects of glucocorticoids (for review, see Wilson, 1988).

The question of the effect of androgens on athletic performance in men is not easy to resolve scientifically for several reasons. (1) The side effects of the drugs at doses taken by athletes are so pronounced as to preclude truly blinded studies of efficacy; (2) only a small subset of users may have a beneficial response, making it difficult to identify the rare responder; and (3) effects on athletic performance become more difficult to assess as the caliber of the athlete increases. For example, a 1% difference in power or speed might be difficult to document between groups but might make a significant difference in the performance of an individual athlete. Regardless of the unresolved scientific issues, many athletes, coaches, and sports physicians believe that the agents do enhance athletic performance; as a consequence, the emphasis in organized sports has shifted from education to mandatory drug testing.

The side effects of androgen abuse still are incompletely understood, in part because many of the agents that are used by athletes are either veterinary drugs or other unapproved derivatives for which human safety data are incomplete. In addition, certain side effects such as peliosis hepatitis may occur only in occasional patients. Finally, adequate long-term toxicity studies have not been performed for any of the agents. As stated above, the side effects of androgens can be separated into virilizing effects, feminizing effects, and toxic effects. All side effects are more common in women and children and hence preclude the use of androgens in these groups by all but a fanatic subset of women athletes. The feminizing and virilizing side effects in adult men are largely reversible, although some effects such as suppression of spermatogenesis may persist for months after the agents are discontinued. Certain long-term toxic effects such as impairment of hepatic function and suppression of high-density lipoprotein concentrations are probably mitigated by the common pattern of intermittent use of the agents. On balance, however, the side effects in men are sufficiently severe to preclude their use for this purpose on medical grounds.

Stimulation of Erythropoiesis. The difference in the hematocrit between men and women is the result of a stimulatory effect of testosterone on the formation of erythropoietin. Castration of men results in a 10% decrease in the mass of red blood cells, a decrease in red-cell diameter, and an increase in osmotic fragility. Occasionally, the anemia may be severe. Administration of androgens to women increases erythropoiesis, and some women develop polycythemia during long-term administration of androgens, as in the treatment of carcinoma of the breast (Shahidi, 1973). In women treated with pharmacological doses of testosterone, the average concentration of hemoglobin increases by 43 g/liter and the hematocrit increases by 0.11 (or 11%). The average increase in hemoglobin is about 10 g/liter in normal men given pharmacological doses of testosterone esters, and red cell volume increases in proportion. Because of these effects, androgens have been used in the treatment of refractory anemias in both men and women. The capacity to enhance erythropoiesis is shared by all active androgens. Some erythropoietin is synthesized by tissues other than the kidney, and the presence of renal tissue is not an absolute requirement for stimulation of erythropoiesis by androgens.

Androgen therapy also has been tried in the anemias associated with failure of the bone marrow, myelofibrosis, and renal failure. Occasional dramatic increases in hemoglobin occur following the administration of androgens to subjects with bone-marrow failure (Azen and Shahidi, 1977). In large numbers of unselected patients treated with androgens, approximately half appear to respond, particularly when the bone marrow is hypoplastic or myelofibrotic. In subjects with Panconi's anemia, an autosomal recessive disorder associated with aplastic anemia, androgen therapy appears to increase life span

by about 4 years (Alter, 1992). What is uncertain, however, is the frequency with which drug administration and therapeutic response are coincidental in subjects with acquired aplastic anemias, in which spontaneous remission can occur during the course of therapy (Branda *et al.*, 1977; Camitta *et al.*, 1979). Additional randomized prospective studies are required before the role of androgens in the routine management of aplastic anemia can be defined, but androgen therapy is reported to result in improved survival rates in aplastic anemias of diverse etiologies (Kaltwasser *et al.*, 1988). When an apparent response occurs, the drug should be stopped temporarily to establish a cause-and-effect relationship between the drug and the apparent response.

Androgens have a minor role in treatment of the anemia of renal failure, particularly because of the availability of recombinant human erythropoietin (see Chapter 53). Androgen-induced increases in concentrations of erythropoietin and hemoglobin are less marked in patients with renal failure than in normal subjects. In addition, the anemia of renal failure may undergo gradual improvement with time following the institution of dialysis and correction of other coexisting causes of anemia. Nevertheless, in most studies androgen therapy results in increases in hemoglobin (10 to 50 g/liter) and in red-blood-cell volume (325 to 350 ml), provided dialysis is adequate and stores of iron and folate are normal (von Hartizsch *et al.*, 1977). Whether or not the benefits of such treatment outweigh the potential adverse effects is unclear, and there is conflicting evidence as to whether or not androgen therapy is synergistic with erythropoietin in subjects with renal failure (Berns *et al.*, 1992).

Hereditary Angioneurotic Edema. In hereditary angioneurotic edema, an autosomal dominant disorder, the plasma contains either a nonfunctional inhibitor of the first component of complement or decreased concentrations of the inhibitor. Thus, there is unopposed activation of the complement cascade, which leads to the generation of factors that enhance the permeability of vessels and cause attacks of angioedema. Several orally active 17 α -alkylated steroids increase the activity of the inhibitor in plasma and restore the components of the complement system that are depleted secondarily. Such therapy usually causes a complete disappearance of symptoms (Cicardi *et al.*, 1991). Steroids, such as *danazol*, that are weak androgens appear to be as effective as, or more effective than, potent androgens (see Table 58-3). Furthermore, the response of men and women to such oral agents appears to be the same. 17 α -Alkylated androgens (but not testosterone or testosterone esters) cause elevations of the concentrations of several plasma glycoproteins that are synthesized in the liver, including some clotting factors and the inhibitor of the first component of complement. The beneficial effect of oral androgens in this disorder is thus likely the result of a side effect of 17 α -alkylated steroids on hepatic function rather than of androgen action *per se* (Barbosa *et al.*, 1971; Gralnick and Rick, 1983). Despite a significant incidence of adverse reactions, long-term danazol therapy appears to be safe in this disorder (Zurlo and Frank, 1990).

Short Stature. Androgens have been used for the management of growth retardation resulting from causes other than pituitary insufficiency. Their administration prior to epiphyseal closure results in an enhancement of linear growth, and the mean advance of height age may be more striking than is skeletal maturation (see Wilson and Griffin, 1980). Such therapy, when given for short periods (6 months or less), has no permanent effects on hypothalamic-pituitary or gonadal maturation. This acceleration of growth may be the result of both an increase in plasma concentration of growth hormone and a direct effect of androgens themselves (Clayton *et al.*, 1988). Whether or not such therapy has a beneficial effect on final adult height in any form

of short stature is not known. For example, treatment of subjects with 45,X gonadal dysgenesis with oral androgens plus growth hormone has no more effect on final height than growth hormone alone (Rosenfeld *et al.*, 1992). Furthermore, administration of androgens to short children prior to the age of 9 years may actually have a deleterious effect on adult height (Bettman *et al.*, 1971). Thus, a role for androgens in the management of any form of short stature other than pituitary dwarfism has not been established.

Carcinoma of the Breast. Testosterone has a palliative effect in some women with carcinoma of the breast. The mechanism is unknown, but the androgen may act as an antiestrogen. The response rates are equivalent to those induced by high doses of estrogen (where an antiestrogenic mechanism also may be operative). No androgen is more efficacious than testosterone, and structural changes in the testosterone molecule that decrease its androgenicity also diminish its effectiveness in breast cancer. Since remission rates are higher with conventional chemotherapy, androgens do not play a major role in the management of this disorder (see Santen *et al.*, 1990).

Osteoporosis. Androgen therapy is effective in treatment of the osteoporosis that complicates androgen deficiency; indeed, the response to hormonal replacement can be dramatic (Isaia *et al.*, 1992). A role for androgens in the treatment of osteoporosis unassociated with male hypogonadism has not been established. See Chapter 61 for more extensive coverage of osteoporosis therapy.

ANTIANDROGENS

Compounds that block the synthesis or action of androgens might be useful in the management of hyperplasia and carcinoma of the prostate, acne, male pattern baldness, virilizing syndromes in women, and precocious puberty in boys and in the inhibition of sex drive in men who are sex offenders.

Inhibitors of Androgen Synthesis. The most effective inhibition of testosterone synthesis is either gonadotropin-releasing hormone (GnRH) itself or an agonist such as *leuprolide* or *gonadorelin*. When such agents are administered continuously, plasma concentrations of LH and testosterone decline so that the net consequence is the induction of a pharmacological (and reversible) castration (see Chapter 55). Such therapy provides a medical alternative to castration for producing androgen deprivation in men with prostate cancer who cannot tolerate stilbestrol (see Santen, 1992).

Antifungal agents of the imidazole class, such as *ketoconazole* and *liarozole*, have as a secondary effect the ability to block cytochrome P450 enzymes involved in steroid hormone biosynthesis (Feldman, 1986). This secondary effect has been turned to therapeutic advantage to induce androgen deprivation in selected patients with prostate cancer (Mahler *et al.*, 1993). Gastrointestinal side effects, a short duration of action, and (with some agents) inhibition of the biosynthesis of adrenal glucocorticoids limit the usefulness of these agents.

Spiroglactone, an aldosterone antagonist (see Chapter 29), acts as a weak inhibitor of the binding of androgen to the androgen receptor but primarily inhibits androgen biosynthesis; in some women with hirsutism, the drug decreases the growth rate and mean diameter of facial hair (Dorrington-Ware *et al.*, 1985). Because it has a tendency to cause metrorrhagia, spiroglactone commonly is given together with an oral contraceptive (Helfer *et al.*, 1988). In efficacy studies, spiroglactone is less effective in improving hirsutism scores than flutamide (Cusan *et al.*, 1994).

5 α -Reductase Inhibitors. Since the conversion of testosterone to

dihydrotestosterone is essential for certain androgen actions, inhibition of 5 α -reductase should selectively block androgen action in those tissues (prostate, hair follicles) in which the continuing production of dihydrotestosterone is essential. The azasteroid *finasteride* (see Table 58-4) is an orally active, competitive inhibitor that preferentially blocks steroid 5 α -reductase 2 but also inhibits enzyme 1 (see Rittmaster, 1994). The agent causes a profound decrease in the concentration of dihydrotestosterone in plasma (Vermeulen *et al.*, 1989) and in the prostate (McConnell *et al.*, 1992) but does not cause a change in plasma testosterone or LH levels.

In men with prostatic hyperplasia, finasteride causes a consistent decrease in prostate size and, in a third of men treated with this agent, improvement in urine flow and symptomatology; it thus provides an alternative to surgery in men with moderate disease manifestations (Stoner, 1992). Finasteride is under trial for the treatment of male pattern baldness, and additional 5 α -reductase inhibitors, including agents specific for steroid 5 α -reductase 1, are under development.

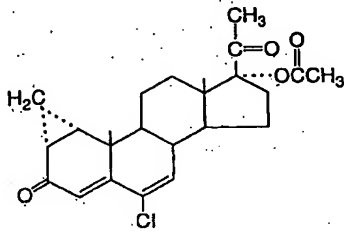
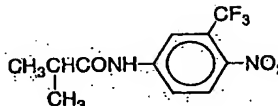
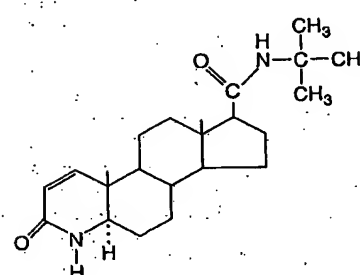
Androgen-Receptor Antagonists. Several drugs are specific antagonists of the binding of androgen to its receptor.

Cyproterone Acetate. Progesterone itself is a weak antiandrogen, and in the search for orally active progestogens, cyproterone acetate was found to be a potent androgen antagonist (Table 58-4). Cyproterone acetate also possesses progestational activity and suppresses the secretion of gonadotropins (Neri, 1976; Neumann, 1982; Neumann and Töpert, 1986). The agent competes with dihydrotestosterone for binding to the androgen receptor (Brown *et al.*, 1981); when given to pregnant animals, cyproterone acetate blocks the actions of androgen in the male fetus and hence induces a form of male pseudohermaphroditism (Hamada *et al.*, 1963). In the castrated animal, the antagonist, at a dose about five times that of testosterone, reduces the androgenic response by about 50%; with larger doses of cyproterone acetate, the antagonism is almost complete (Neumann *et al.*, 1970).

The administration of 100 mg per day of cyproterone acetate to normal young men causes a 50% decrease in plasma concentrations of LH and FSH and a 75% decrease in plasma testosterone; the effects of the drug result both from inhibition of testosterone production and from interference with androgen action (Knuth *et al.*, 1984). The agent has been used for the treatment of acne, male pattern baldness, hirsutism, and virilizing syndromes (see Neri, 1976; Neumann, 1982; Neumann and Töpert, 1986). It also has been tried in the treatment of precocious puberty (Kauli *et al.*, 1976) and prostatic hyperplasia and carcinoma (see Namer, 1988) and to inhibit libido in men with severe deviations of sexual behavior (Laschet *et al.*, 1967). Although cyproterone acetate is still under investigation, the agent has orphan-drug status in the United States for the treatment of severe hirsutism.

Flutamide. Flutamide (see Table 58-4) is a nonsteroidal antiandrogen that is devoid of other hormonal activity; it probably acts after conversion *in vivo* to 2-hydroxyflutamide, which is a potent competitive inhibitor of binding of dihydrotestosterone to the androgen receptor (see Neri, 1976). In the mature rat, the agent causes regression of androgen target tissues such as the prostate and seminal vesicles, and, by blocking the inhibitory feedback of testosterone on LH production, results in a profound increase in plasma concentrations of LH and testosterone (Marchetti and Labrie, 1988). Similar effects have been observed in men treated with 750 mg of flutamide per day (Knuth *et al.*, 1984). The predominant pituitary effect of flutamide appears to be enhancement of the frequency of pulses of LH secretion (Urban *et al.*, 1988). Thus, while the drug is an effective antiandrogen *in vitro*, the rise in plasma testosterone serves to limit its antiandrogenic effects. As a consequence, flutamide is most useful

Table 58-4
Some Androgen Receptor Antagonists and 5 α -Reductase Inhibitors

NONPROPRIETARY NAME AND TRADE NAME	CHEMICAL STRUCTURE	DOSAGE AND INDICATIONS
Cyproterone Acetate ANDROCUR		For severe hirsutisms; investigational
Flutamide EULEXIN		Capsules; 750 mg/day in combination with GnRH agonistic analogs; for metastatic prostatic carcinoma
Finasteride PROSCAR		Tablets; 5 mg/day; for benign prostatic hyperplasia

to inhibit the action of adrenal androgens in castrated men or in men receiving GnRH continuously (GnRH blockade) or in situations in which LH production is not under predominant control by androgen (as in normal women).

The principal clinical application of flutamide to date is in the treatment of prostatic cancer, usually in conjunction with GnRH blockade or estrogen (*see Geller et al., 1988*). Flutamide also has been used experimentally in combination with an oral contraceptive for the treatment of hirsutism in women (*see Cusan et al., 1994*). If flutamide crosses the placenta, it would be expected to produce male pseudohermaphroditism, as is the case for cyproterone acetate; hepatotoxicity, including progressive liver failure, limits its usefulness (Dankoff, 1992; Wysowski *et al.*, 1993). For the treatment of metastatic prostatic carcinoma, flutamide should be administered in conjunction with a GnRH antagonist such as leuprolide.

Additional antiandrogens such as nilutamide and casodex are under investigation.

MALE CONTRACEPTIVES

There are many requirements of the ideal contraceptive drug: simplicity, acceptability, reversibility, lack of toxicity, and, of course, efficacy. Although all of these criteria have not been attained in the

oral contraceptives for women, the agents discussed in Chapter 57 come close. The lack of development of effective, safe contraceptives for men is due principally to the fact that it is difficult to inhibit spermatogenesis completely; men have fathered children even when sperm counts are lowered by 99% (to values of approximately 1 million per milliliter) (*see Diller and Hembree, 1977; Bialy and Patanelli, 1981; Reyes and Chavarria, 1981*).

A variety of compounds, in addition to the antiandrogens discussed above, can inhibit spermatogenesis. They include antineoplastic agents, cadmium, nitrofurans, α -chlorhydrin, and dinitro-pyrrole. However, the irreversible effects of some and the toxicity of many preclude their clinical use.

Gossypol, a phenolic compound extracted from the cotton plant, reduces sperm density to less than 4 million/ml in 99.9% of men and impairs sperm motility. Normal sperm density is restored within several months of discontinuation of the drug (*see Lawrence, 1981*). Unfortunately, administration of gossypol causes hypokalemia and weakness; diarrhea, edema, dyspnea, neuritis, and paralysis are observed after higher doses are taken. These effects also are seen in cottonseed poisoning.

Gonadal steroids can suppress secretion of FSH and LH, which are required for spermatogenesis and the synthesis of testosterone by the testes (*see Chapters 55 and 57*). While estrogens and progestins are effective contraceptives in men, suppression of testosterone de-

creases both libido and potency; gynecomastia also may occur. The injection of testosterone esters in supraphysiologic doses inhibits gonadotropin secretion and spermatogenesis and is under trial as a contraceptive regimen.

Another approach to male contraception has involved the concomitant administration of an androgen and progestin (Brenner *et al.*, 1975). The rationale for this combination was to enhance the suppression of gonadotropin secretion and spermatogenesis and to prevent alteration of accessory sexual structures (Brenner and DeKretser, 1976). Unfortunately, not all subjects develop azoosper-

mia, and sperm counts are suppressed only after several months of treatment. A similar period of time is required for the recovery of spermatogenesis when the drugs are stopped.

Potent agonists and antagonists of GnRH can inhibit secretion of gonadotropins and can be administered together with testosterone; while this regimen does not result in uniform azoospermia, at least in some studies it does appear to cause consistent infertility (Pavlou *et al.*, 1991). All the methods described above for contraception in the male remain investigational.

For further discussion of diseases associated with altered testicular function, see Chapter 339 in *Harrison's Principles of Internal Medicine*, 13th ed., McGraw-Hill, New York, 1994.

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Pharmacodynamics of Selective Androgen Receptor Modulators

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ABSTRACT

The present study aimed to identify selective androgen receptor modulators (SARMs) with *in vivo* pharmacological activity. We examined the *in vitro* and *in vivo* pharmacological activity of four chiral, nonsteroidal SARMs synthesized in our laboratories. In the *in vitro* assays, these compounds demonstrated moderate to high androgen receptor (AR) binding affinity, with K_i values ranging from 4 to 37 nM, and three of the compounds efficaciously stimulated AR-mediated reporter gene expression. The compounds were then administered subcutaneously to castrated rats to appraise their *in vivo* pharmacological activity. Androgenic activity was evaluated by the ability of these compounds to maintain the weights of prostate and seminal vesicle, whereas levator ani muscle weight was used as a measure of anabolic activity. The maximal response (E_{max}) and dose for half-maximal effect (ED_{50}) were determined for each

compound and compared with that observed for testosterone propionate (TP). Compounds S-1 and S-4 demonstrated *in vivo* androgenic and anabolic activity, whereas compounds S-2 and S-3 did not. The activities of S-1 and S-4 were tissue-selective in that both compounds stimulated the anabolic organs more than the androgenic organs. These two compounds were less potent and efficacious than TP in androgenic activity, but their anabolic activity was similar to or greater than that of TP. Neither S-1 nor S-4 caused significant luteinizing hormone or follicle stimulating hormone suppression at doses near the ED_{50} value. Thus, compounds S-1 and S-4 were identified as SARMs with potent and tissue-selective *in vivo* pharmacological activity, and represent the first members of a new class of SARMs with selective anabolic effects.

Endogenous androgens play crucial physiological roles in establishing and maintaining the male phenotype (George and Wilson, 1986; Mooradian et al., 1987). Their actions are essential for the differentiation and growth of male reproductive organs, initiation and regulation of spermatogenesis, and control of male sexual behavior. In addition, androgens are important for the development of male characteristics in certain extragenital structures such as muscle, bone, hair, larynx, skin, lipid tissue, and kidney (Takeda et al., 1990). In females, the precise physiological roles of androgens are not completely understood, but the age-related decline in circulating androgen levels has been linked to symptoms such as decreased libido and sexuality, lack of vigor, diminished well

being, and loss of bone mineral density in postmenopausal women (Davis and Burger, 1996; Davis, 1999a,b).

Synthesized steroidal androgens, due to their ability to mimic the actions of their endogenous counterparts, have been used clinically as valuable therapeutic agents to target a variety of male and female disorders resulting from androgen deficiency. The principle clinical indication of androgens is as replacement therapy for hypogonadal men (Conway et al., 1988; Wu, 1992). Other documented clinical uses of androgens include delayed puberty in boys, anemias, primary osteoporosis, hereditary angioneurotic edema, endometriosis, estrogen receptor-positive breast cancer, and muscular diseases (Wu, 1992; Bagatell and Bremner, 1996; Nieschlag, 1996; Bhasin and Tenover, 1997). Also, androgens have been investigated as hormone replacement therapy for aging men and for regulation of male fertility (Wu, 1992; Tenover, 1997).

Since the discovery of the therapeutic benefits of testosterone in the 1930s, a variety of androgen preparations have been introduced and tested clinically. Unfortunately, virtu-

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ABBREVIATIONS: AR, androgen receptor; SARM, selective androgen receptor modulator; TP, testosterone propionate; PEG 300, polyethylene glycol 300; FSH, follicle stimulating hormone; LH, luteinizing hormone; ANOVA, analysis of variance; DHT, dihydrotestosterone; AST-SGOT, serum glutamicoyaloacetic transaminase; ALT-SGPT, serum glutamic pyruvic transaminase.

ally all currently available androgen preparations have severe limitations (Wu, 1992; Bhasin and Bremner, 1997). Unmodified testosterone is impractical for oral administration due to its low systematic bioavailability (Handelsman et al., 1990). Testosterone esters (e.g., testosterone propionate and testosterone enanthate) are presently the most widely used testosterone preparations, usually administered by intramuscular injection in oil vehicles (Snyder and Lawrence, 1980; Velazquez and Bellabarba Arata, 1998). A prolonged duration of action is achievable with these esters. However, they produce highly variable testosterone levels. 17 α -Alkylated testosterone (e.g., methyltestosterone and oxandrolone) can be given orally. Nevertheless, they often cause unacceptable hepatotoxicity and are less efficacious; hence, they are not recommended for long-term androgen therapy (Heywood et al., 1977; Ishak and Zimmerman, 1987; Velazquez and Bellabarba Arata, 1998). Another common concern about steroidal androgens is the undesirable effects resulting from the cross-reactivity of the androgens or their in vivo metabolites with steroid receptors other than the androgen receptor (AR) (Wilson et al., 1980; Bhasin and Bremner, 1997).

During studies with affinity ligands for the AR, our group discovered a group of nonsteroidal androgens that are structural derivatives of bicalutamide and hydroxyflutamide, two known antiandrogens (Dalton et al., 1998; Mukherjee et al., 1999). Other laboratories have also reported the identification of nonsteroidal compounds that possess androgen activity (Dalton et al., 1998; Hamann et al., 1999; Negro-Vilar, 1999). The discovery of these nonsteroidal androgens offers an opportunity for the development of a new generation of selective androgen receptor modulators (SARMs) superior to current steroidal androgens. Theoretically, SARMs are advantageous over their steroidal counterparts in that they can obtain better receptor selectivity and allow greater flexibility in structural modification. Thus, SARMs can potentially avoid the undesirable effects caused by receptor cross-reactivity and achieve superior pharmacokinetic properties.

Subsequent to our initial discovery of several nonsteroidal androgens, our laboratories designed and synthesized multiple series of nonsteroidal compounds, and explored the structure-activity relationships for androgenic and anabolic activities, both in vitro and in vivo (He et al., 2002; Yin et al., 2003a,b). According to results from these structure-activity relationship studies, we designed a group of novel nonsteroidal compounds (Fig. 1) that were structurally optimized. We report herein the results of our studies to examine the in vitro AR binding affinity and the androgenic and anabolic activities of these new compounds in an animal model. Two potent and tissue-selective SARMs were identified from these structurally similar compounds, and they are members of a promising new class of drug candidates for further development.

Materials and Methods

Materials. The *S*-isomers of compounds 1, 2, 3, and 4, and the *R*-isomer of compound 1 were synthesized in our laboratories (synthetic procedures will be reported separately). The purities of these compounds were greater than 99%, as determined by high-performance liquid chromatography. Testosterone propionate (TP), polyethylene glycol 300 (PEG 300, reagent grade), and dimethyl sulfoxide

(reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl alcohol USP was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). Alzet osmotic pumps (model 2002) were purchased from Alza (Palo Alto, CA).

In Vitro Pharmacological Activity. Cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats (about 250 g). The binding affinity of compounds 1, 2, 3, and 4 to the AR preparation was determined and analyzed as described previously (Mukherjee et al., 1996, 1999). The ability of the compounds to influence AR-mediated transcriptional activation was examined using a cotransfection system, as described previously (Yin et al., 2003a). Transcriptional activation was measured using a single concentration (10 nM) of the indicated compound and reported as a percentage of the transcriptional activation observed for 1 nM DHT.

Animals. Male Sprague-Dawley rats, weighing 90 to 100 g, were purchased from Harlan Bioproducts for Science (Indianapolis, IN). The animals were maintained on a 12-h light/dark cycle with food and water available ad libitum. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Study Design. Animals were randomly distributed into 30 groups, with five rats per group. Treatment groups are described in Table 1. One day before the start of drug treatment, animals in groups 2 through 30 were surgically castrated. After 24 h of recovery, Alzet osmotic pumps (model 2002) prefilled with a designated solution (Table 1) were implanted subcutaneously in the scapular region of castrated animals. Drug solutions used to fill the osmotic pumps were prepared using aseptic techniques. For solutions of nonsteroidal compounds and low-dose (0.1 mg/day or lower) solutions of TP, drugs were first dissolved in minimal amounts of ethanol and then diluted to final concentrations with PEG 300 (this vehicle is designated as vehicle 1). Because higher doses of TP could not be completely solubilized in the above-mentioned vehicle, TP solutions for 0.3, 0.5, and 0.75 mg/day were prepared by dissolving the drug in a mixture of ethanol and dimethyl sulfoxide and adjusting with PEG 300 to the desired final volume (this vehicle was designated as

TABLE 1
Animal groups and experimental design

Group	Castration	Drug	Dose mg/day	No. of Animals
1	No	None	None	5
2	Yes	None	Vehicle 1 only	5
3	Yes	Testosterone	0.03	5
4	Yes	Testosterone	0.05	5
5	Yes	Testosterone	0.1	5
6	Yes	Testosterone	0.3	5
7	Yes	Testosterone	0.5	5
8	Yes	Testosterone	0.75	5
9	Yes	R-1	1.0	5
10	Yes	S-1	0.1	5
11	Yes	S-1	0.3	5
12	Yes	S-1	0.5	5
13	Yes	S-1	0.75	5
14	Yes	S-1	1.0	5
15	Yes	S-2	0.1	5
16	Yes	S-2	0.3	5
17	Yes	S-2	0.5	5
18	Yes	S-2	0.75	5
19	Yes	S-2	1.0	5
20	Yes	S-3	0.1	5
21	Yes	S-3	0.3	5
22	Yes	S-3	0.5	5
23	Yes	S-3	0.75	5
24	Yes	S-3	1.0	5
25	Yes	S-4	0.1	5
26	Yes	S-4	0.3	5
27	Yes	S-4	0.5	5
28	Yes	S-4	0.75	5
29	Yes	S-4	1.0	5
30	Yes	None	Vehicle 2 only	5

vehicle 2). Due to the limited solubility of TP, two osmotic pumps were used in each animal to deliver TP at 0.5 and 0.75 mg/day. One osmotic pump was used in each animal for other groups.

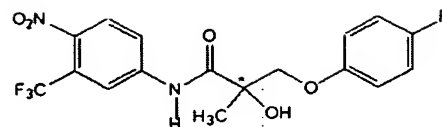
After 14 days of drug treatment, rats were weighed, anesthetized, and sacrificed. Blood samples were collected by venipuncture of the abdominal aorta. For each animal, a whole blood sample and a serum sample were used for complete blood count and chemistry profile analyses, and a portion of the blood was centrifuged to prepare plasma. Plasma samples from selected groups were analyzed for FSH, LH, GH, AST-SGOT, ALT-SGPT, cholesterol, high-density lipoprotein, and triglyceride. Plasma samples for these analyses were collected from a separate group of animals in the case of the TP-treated groups, using dose rates of 0.1, 0.3, 0.5, 0.75, and 1 mg/day. The ventral prostates, seminal vesicles, levator ani muscle, liver, kidneys, spleen, lungs, and heart were removed; cleared of extraneous tissue; weighed; and placed in vials containing 10% neutral buffered formalin. Preserved tissues were subjected to histopathological analysis. Osmotic pumps were removed from animals to check for correct pump operation.

Data Analyses. The weights of all organs were normalized to body weight, and analyzed for any statistically significant differences between groups using single-factor ANOVA with the α value set a priori at $p < 0.05$. The weights of prostates and seminal vesicles were used as indices for evaluation of androgenic activity, and the levator ani muscle weight was used to evaluate the anabolic activity. Statistical analyses of parameters from complete blood count or serum chemical profiling, wherever applicable, were performed by single-factor ANOVA with the α value set a priori at $p < 0.05$. For compounds demonstrating full-range dose-response relationships in any of the measured parameters, the maximal response produced by the compound (E_{max}) and the dose rate that induced 50% of the maximal response (ED_{50}) were obtained by nonlinear regression analysis using WinNonlin (version 3.1; Pharsight Corporation, Mountain View, CA) and the sigmoid E_{max} model. The E_{max} value indicated the efficacy of each compound, whereas the ED_{50} indicated its potency. The relative efficacy of each compound to TP was defined as the ratio of (E_{max} of the compound) to (E_{max} of TP). The relative potency was defined as the ratio of (ED_{50} of TP) to (ED_{50} of the compound).

Results

The *in vitro* AR binding of the *R*-isomer of compound 1 (designated as *R*-1) and the *S*-isomers of compounds 1, 2, 3, and 4 (designated as *S*-1, *S*-2, *S*-3, and *S*-4, respectively) was examined with a radioligand competitive binding assay. *R*-1 demonstrated poor AR binding affinity ($K_i = 225 \pm 15$ nM), whereas *S*-1, *S*-2, *S*-3, and *S*-4 bound to the AR with moderate to high affinity, with K_i values ranging from 4 to 37 nM (Fig. 1). Next, the ability of these compounds to stimulate AR-mediated transcription was determined in an *in vitro* cotransfection system. At a concentration of 10 nM, compounds *S*-3 and *S*-4 stimulated AR-mediated transcription to 75 and 93%, respectively, of that observed for 1 nM DHT, whereas compounds *S*-1 and *S*-2 demonstrated lesser stimulation (i.e., 43 and 9.7%, respectively). Given previous studies in our laboratories demonstrating that *in vitro* cotransfection models poorly predict *in vivo* pharmacological activity (Yin et al., 2003a), we then examined the androgenic and anabolic activities of these nonsteroidal compounds in a castrated rat model after 14 days of drug administration. *R*-1 was included as a negative control. TP, at increasing doses, was used as the positive control for anabolic and androgenic effects.

In accordance with literature reports (Saksena and Chaudhury, 1970; Teutsch et al., 1994; Battmann et al.,



Name	Isomer	R	K _i (nM)	Activation (% of 1 nM DHT)
R-1	R	F	225 ± 15	N.D.
S-1	S	F	6.1 ± 0.2	43 ± 2.8
S-2	S	COCH ₃	37 ± 2.4	9.7 ± 1.5
S-3	S	COC ₂ H ₅	6.1 ± 0.1	75 ± 11
S-4	S	NHCOC ₂ H ₅	4.0 ± 0.7	93 ± 7.0

Fig. 1. Chemical structures and *in vitro* pharmacological activity of nonsteroidal AR ligands. Cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats. The AR binding affinity was determined and analyzed as described previously (Mukherjee et al., 1996, 1999). The ability of the compounds to influence AR-mediated transcriptional activation was examined using a cotransfection system, as described previously (Dalton et al., 1998). Transcriptional activation was measured using a single concentration (10 nM) of the indicated compound and reported as a percentage of the transcriptional activation observed for 1 nM DHT.

1998), we observed significant decreases in the weights of prostate, seminal vesicles, and levator ani muscle in castrated, vehicle-treated rats (Figs. 2-5). The weights of the

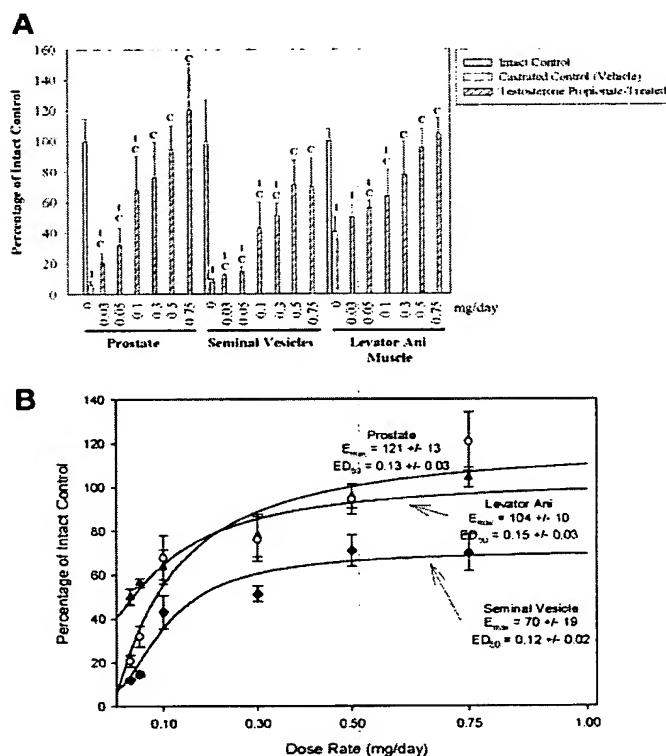


Fig. 2. A, assay for androgenic and anabolic activity of TP in castrated immature rats. One day after castration, immature rats received 1 mg/day of TP via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5$ /group). The letters "I" and "C" above the error bars indicate a significant difference between the group and the intact control group or castrated control group, respectively, as tested by single-factor ANOVA ($p < 0.05$). B, dose-response relationships of TP. E_{max} and ED_{50} values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{max} model.

prostate, seminal vesicles, and levator ani muscle in castrated rats were 6.2, 8.1, and 40.9%, respectively, of those in intact animals. The reduction in masses of these androgen-targeted organs in castrated animals is the result of ablation of endogenous androgen production (Saksena and Chaudhury, 1970). Exogenous administration of TP, an androgenic and anabolic steroid, increased weights of the prostate, seminal vesicles, and levator ani muscle in castrated rats (Fig. 2). The increases in organ weights induced by TP were dose rate-dependent.

Figure 3 shows that compound S-1 had no significant effect on prostate, seminal vesicles, and levator ani muscle in castrated animals at 0.1 and 0.3 mg/day, but significantly stimulated the growth of these organs at higher doses. The weights of prostate, seminal vesicles, and levator ani muscle were maximally restored by S-1 to 14.9, 13.4, and 74.3%, respectively, of those in intact animals. The ED_{50} values of S-1 in prostate, seminal vesicle, and levator ani muscle, as obtained by nonlinear regression analysis of dose-response relationships, were 0.42 ± 0.04 , 0.38 ± 0.26 , and 0.44 ± 0.01 mg/day, respectively (Fig. 3B; Table 2), corresponding to 1.63, 1.47, and 1.70 mg/kg, respectively, based on the mean body weight of S-1-treated animals at the end of the study. The

elevations in organ weights by S-1 demonstrated its androgenic and anabolic activities in animals. In comparison to TP, corresponding dose rates of S-1 induced significantly smaller increases in the weight of the prostate and seminal vesicles but a similar degree of increase in levator ani muscle weight (compare Fig. 2A with 3A). This result denoted the tissue selective androgenic and anabolic activity of S-1 in rats. The selectivity was also demonstrated by its relative efficacy compared with TP (Table 2). The relative efficacy in maintaining levator ani muscle weight was 0.72, much higher than the relative efficacies in maintaining prostate and seminal vesicle weights, which were less than 0.20.

Despite their high AR binding affinity, compounds S-2 and S-3 failed to exert any significant effect on the weights of prostate, seminal vesicles, and levator ani muscle in castrated animals, with dose rates up to 1 mg/day (Fig. 4). This suggests that rapid metabolism or clearance of these compounds led to lower plasma concentrations of these drugs, and thus no pharmacological activity. Likewise, compound R-1 (the stereoisomer of S-1), at 1 mg/day, produced no apparent effect on the weights of prostate, seminal vesicles, and levator ani muscle in castrated animals, demonstrating the stereoselective pharmacological action of these compounds.

Compound S-4 (Fig. 5) caused dose-dependent stimulation of growth in prostate, seminal vesicles, and levator ani muscle, with their weights in castrated animals being maximally promoted to 33.8, 28.2, and 101% of intact controls, respectively. Nonlinear regression analysis of dose-response relationships showed that the ED_{50} values of S-4 were 0.43 ± 0.01 , 0.55 ± 0.02 , and 0.14 ± 0.01 mg/day in prostate, seminal vesicles, and levator ani muscle, respectively (Fig. 5B; Table 2), corresponding to 1.62, 2.07, and 0.53 mg/kg, respectively, based on the mean body weight of S-4-treated animals at the end of the study. These results clearly revealed the androgenic and anabolic activities of S-4 in animals. In particular, S-4 exhibited potent and efficacious anabolic activity, as indicated by its ability to fully maintain the levator ani muscle weight in castrated animals at the same level as intact controls, at a dose rate as low as 0.3 mg/day (Fig. 5A). The relative potency and efficacy of S-4 in androgenic tissues were less than 0.3 and 0.4, respectively, compared with 1 for TP, whereas its relative potency and efficacy in the levator ani muscle was 1.07 and 0.97, respectively, compared with 1 for TP (Table 2).

Table 2 compares the androgenic and anabolic activities of S-1 and S-4, two compounds that exhibited *in vivo* functional activity in the present study, with those of TP. The efficacy for androgenic activity of S-4 (as indicated by relative efficacies in prostate and seminal vesicle) was about twice that of S-1, but the potency for androgenic activity (as indicated by relative potencies in prostate and seminal vesicle) was similar between these two compounds. As to anabolic activity, S-4 displayed much higher efficacy (as indicated by relative efficacy in levator ani muscle) and 2-fold greater potency (as indicated by relative potency in levator ani muscle) than S-1. These results suggest the greater selectivity of S-4 toward the anabolic target organ.

We also determined the serum levels of LH and FSH in animals that received S-1 and S-4, and compared them with the levels of these hormones observed in the intact, castrated, or TP-treated animals. As shown in Table 3, castration led to a significant elevation in FSH and LH levels, compared with

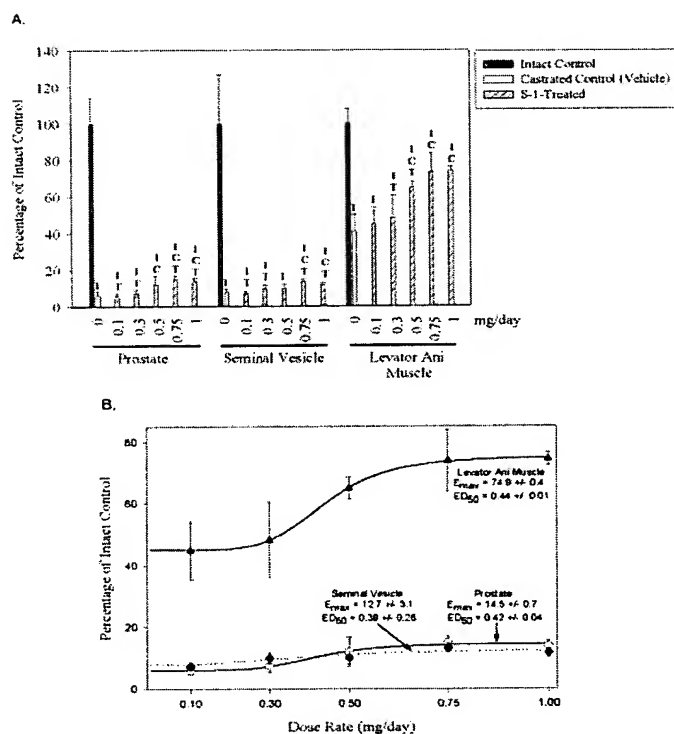


Fig. 3. A, assay for androgenic and anabolic activity of S-1 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of S-1 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5/\text{group}$). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$); B, dose-response relationships of S-1. E_{max} and ED_{50} values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{max} model.

TABLE 2

Comparison of androgenic and anabolic activities of S-1 and S-4 to TP

Organs	Treatment	E_{\max} (Percentage of Intact Control)	Relative Efficacy	ED ₅₀ mg/day	Relative Potency
Androgenic Prostate	TP	120.6 ± 13.4	1.00	0.13 ± 0.03	1.00
	S-1	14.5 ± 0.7	0.12	0.42 ± 0.04	0.31
	S-4	35.2 ± 0.4	0.29	0.43 ± 0.01	0.30
Seminal vesicle	TP	70.0 ± 18.8	1.00	0.12 ± 0.02	1.00
	S-1	12.7 ± 3.1	0.18	0.38 ± 0.26	0.32
	S-4	28.5 ± 0.8	0.40	0.55 ± 0.02	0.22
Anabolic Levator ani muscle	TP	104.2 ± 10.1	1.00	0.15 ± 0.03	1.00
	S-1	74.9 ± 0.4	0.72	0.44 ± 0.01	0.34
	S-4	101.0 ± 1.0	0.97	0.14 ± 0.01	1.07

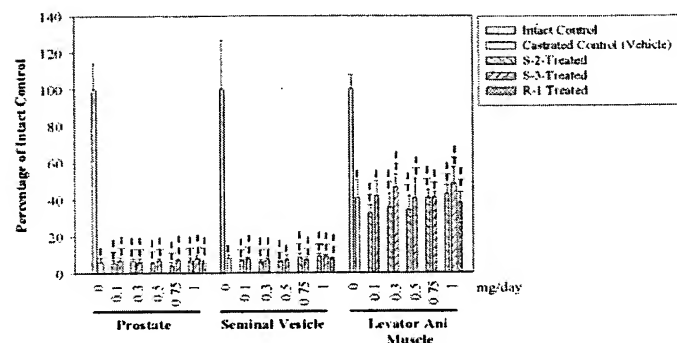


Fig. 4. Assay for androgenic and anabolic activity of S-2, S-3, and R-1 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of S-2, S-3, or R-1 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5$ /group). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$).

intact animals. TP showed no dose-dependent effect on castration-induced change in FSH, but partially inhibited the castration-induced increase in LH levels at higher doses. The activities of S-4 on LH and FSH were similar to those produced by TP. S-1 and S-4 partially suppressed LH production at dose rates of 0.5 mg/day or higher. However, it is important to note that S-1 and S-4 did not suppress LH production at the dose levels needed to produce the desired pharmacological effects in the levator ani muscle or prostate. Interestingly, S-1 also partially suppressed FSH production at dose rates of 0.5 mg/day or higher. The FSH suppression noted at higher doses of S-1 suggested that this compound might interact with other steroid receptors, most probably progesterone receptors, in addition to the AR. Although statistically significant differences were noted in some instances, GH, AST-SGOT, ALT-SGPT, and serum lipids (including cholesterol, high-density lipoprotein, and triglyceride) were all within normal ranges for drug-treated animals. No drug- or dose-related changes in these indices were observed.

We also examined the effects of all compounds on total body weight and the weights of a variety of nonreproductive organs, including liver, heart, kidney, spleen, and lungs of treated animals. None of the compounds led to a dose-related change in these weights (data not shown). To further check for any signs of acute toxicity in animals from the studied

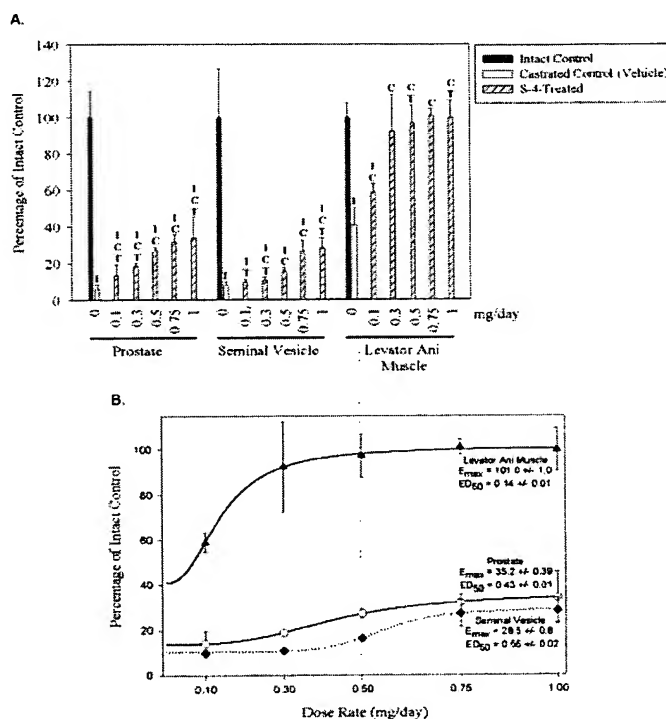


Fig. 5. A, assay for androgenic and anabolic activity of S-4 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of S-4 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5$ /group). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$). B, dose-response relationships of S-4. E_{\max} and ED₅₀ values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{\max} model.

compounds, complete diagnostic hematology studies of compound-treated animals were also performed. No drug- or dose-related changes were observed in any of the hematology diagnostic indices. These data suggest that all compounds manifested no acute toxicity during treatment.

Discussion

With in vitro AR binding and transcription activation assays, our laboratories previously identified a group of potent

TABLE 3
Effects of S-1 and S-4 on serum LH and FSH levels

	FSH	LH
	ng/ml	ng/ml
Normal Range	5.6–20	0.15–0.7
Treatment		
None (intact)	7.1 ± 1.2	0.27 ± 0.08
None (castrated)	30 ± 3 ^I	5 ± 2 ^I
TP (mg/day)		
0.1	26 ± 6 ^I	4 ± 4 ^I
0.3	23 ± 6 ^I	2 ± 1 ^{I,C}
0.5	30 ± 7 ^I	5 ± 2 ^I
0.75	22 ± 5 ^{I,C}	1.4 ± 0.7 ^{I,C}
1.0	24 ± 8 ^I	2 ± 1 ^{I,C}
S-1 (mg/day)		
0.1	28 ± 7 ^I	5 ± 2 ^I
0.3	27 ± 5 ^I	3 ± 1 ^I
0.5	22 ± 3 ^{I,C}	1.7 ± 0.3 ^{I,C,T}
0.75	20 ± 5 ^{I,C}	1.0 ± 0.7 ^C
1	23 ± 3 ^{I,C}	1.6 ± 0.7 ^{I,C}
S-4 (mg/day)		
0.1	27 ± 5 ^I	4 ± 1 ^I
0.3	26 ± 1 ^I	3.7 ± 0.9 ^I
0.5	26 ± 8 ^I	2.4 ± 0.9 ^{I,C}
0.75	31 ± 5 ^{I,T}	3 ± 1 ^I
34 ± 5 ^{I,T}	2.4 ± 0.4 ^{I,C}	

I, C, and T: significantly different ($p < 0.05$) from intact, castrated, and corresponding dose rate of TP-treated animals, respectively, as analyzed by single-factor ANOVA.

and efficacious nonsteroidal androgens that are structurally related to antiandrogen pharmacophores (Yin et al., 2003a). However, in vivo studies in a rat model with one of these nonsteroidal androgens, acetothiolutamide, failed to show androgenic activity (Yin et al., 2003b). Subsequent pharmacokinetic and metabolism studies in rats demonstrated that the lack of in vivo androgenic activity of acetothiolutamide in the pharmacology study was caused by its insufficient plasma exposure, which in turn resulted from its extensive hepatic degradation. Also, we found that oxidation at the sulfur linkage position was one major metabolic pathway for acetothiolutamide in rats, and that this oxidation likely produced deactivated or even antagonizing metabolites (Yin et al., 2003b). Considering these facts, we proposed to modify the linkage sulfur atom to block the oxidation at this position, thereby reducing the overall hepatic metabolism. As a result, a series of novel molecules that carry an ether linkage instead of a thio linkage in the structure were designed and synthesized. The present studies demonstrated that two of these ether-bearing molecules, S-1 and S-4, were androgen receptor modulators with tissue-selective activity in animals.

Despite structural similarities, this series of ether-carrying compounds exhibited diverse in vitro and in vivo activity profiles. The S-isomers of compounds 1, 2, 3, and 4 displayed moderate to high binding affinity for the AR, whereas the R-isomer of compound 1 had poor receptor binding. This finding was consistent with our previous observation regarding the stereoselective AR binding of nonsteroidal ligands (Mukherjee et al., 1996, 1999). Compounds S-1, S-3, and S-4 were further characterized as AR agonists with the in vitro cotransfection assay. The failure of S-2, a moderate AR binder, to stimulate AR-mediated gene transcription confirmed our previous finding that high receptor binding affinity is a prerequisite for agonist activity (Yin et al., 2003a). When tested in the castrated rat model, S-1 and S-4 demonstrated potent in vivo functional activity, and compounds S-2 and S-3 were inactive. Specifically, S-4 produced the greatest

androgenic and anabolic activity in animals, with anabolic activity greater than that of TP. S-1 had a similar degree of anabolic activity as TP, but had much less androgenic activity. Interestingly, the ED₅₀ values for S-1 in prostate, seminal vesicle, and levator ani muscle were approximately the same (i.e., about 0.4 mg/day), whereas S-4 demonstrated more than 2-fold greater potency in levator ani muscle compared with prostate and seminal vesicle, as indicated by the ED₅₀ values (Table 2). The distinction in functional activities in vivo among the four structurally related compounds could be caused by difference in any of numerous factors, including intrinsic activity, in vivo disposition and metabolism, or intracellular signaling pathway. Further studies to explore the physicochemical, physiological, and cellular/molecular determinants for nonsteroidal androgenic and anabolic activity will lead to insights into the mechanism of action of these nonsteroidal agents, and thereby provide a basis for future structural optimization.

The in vitro cotransfection assay is generally regarded as a valuable tool for screening of nonsteroidal AR ligands. With this assay, compounds S-1, S-3, and S-4 were successfully identified as potential AR agonists. However, as demonstrated in the animal study, S-3 did not show any measurable in vivo functional activity. Thus, the observation of in vitro agonist activity in the cotransfection assay can be but is not always predictive of in vivo activity. The pharmacological activity in vivo is determined not only by the ability of the compound to interact with the receptor, but also limited by complicated factors governing the accessibility of the compound to the effect site, such as disposition and metabolism. To fully predict the in vivo behavior and understand the structure-activity relationships, it is necessary to perform further studies examining the pharmacokinetics and metabolism of the compound. As a result of these and other studies, we abandoned use of the in vitro cotransfection assay in favor of in vivo pharmacologic assessment for discovery of SARMS.

The tissue-selective anabolic activity exhibited by S-1 and S-4 validated the feasibility of developing SARMS as a new generation of androgens. The possible mechanisms underlying the tissue-selectivity of these agents could be tissue-specific recruitment of cofactors/corepressors during the AR signaling pathway, or very likely for our nonsteroidal ligands, their distinct in vivo disposition from testosterone and its ester derivatives. The effects of testosterone in certain tissues, including most accessory reproductive organs and skin, are amplified through local conversion to DHT, the more potent bioactive form, by 5 α -reductase (Mooradian et al., 1987). Nevertheless, testosterone exerts direct effects in the testis, skeletal muscles, and bone (Mukherjee et al., 1996). For nonsteroidal ligands, their actions in accessory reproductive organs such as prostate would not be amplified as they are for testosterone; therefore, such a nonsteroidal androgen with equivalent activity to testosterone on bone and muscle would likely have less activity on prostate or other accessory reproductive organs than testosterone.

Compounds S-1 and S-4 are the first nonsteroidal androgens with in vivo functional activity among our series of compounds. More significantly, the discovery of these two in vivo functional drug candidates represents a major progress toward the development of therapeutically useful SARMS. SARMS, like the clinically available selective estrogen receptor modulators, would offer unique therapeutic advantages

over their steroidal counterparts. The tissue selectivity of these agents offers an exciting opportunity to differentially regulate the androgen effects in various target tissues, thus minimizing the interference to normal physiological processes while targeting desirable therapeutic goals. For example, SARMs with potent anabolic activity but minimal androgenic activity would be ideal for the treatment of patients who bear muscular diseases (such as sarcopenia or trauma-induced muscle wasting) but are contraindicated for androgenic stimuli (such as for aging population or prostate cancer patients). In perspective, not only could SARMs be used as superior alternatives to current steroidal androgens in therapy of male hypogonadism but also they could expand the scope of androgen therapy to include wasting syndromes, aging-related disorders due to declined androgen levels, male fertility regulation, and other androgen deficiency-related diseases.

In summary, the present studies examined the *in vitro* and *in vivo* activity profiles of a series of novel nonsteroidal AR ligands, among which two were identified as *in vivo* functional androgens with selective anabolic activity. These SARMs, with many advantages over current steroidal androgen preparations, implicate potential therapeutic significance in a scope of androgen-deficiency related disorders. Continued studies in our laboratories will focus on preclinical and clinical development of identified SARMs and further optimization of chemical structures based on understanding the mechanisms underlying nonsteroidal androgenic and anabolic activities.

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Update and Future of Systemic Acne Treatment

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Key Words

Acne · Therapy · Update · Future · Review

Abstract

Systemic treatment is required in patients with moderate-to-severe acne, especially when acne scars start to occur. Antibiotics with anti-inflammatory properties, such as tetracyclines (oxytetracycline, tetracycline chloride, doxycycline, minocycline and lincycline) and macrolide antibiotics (erythromycin and azithromycin) are the agents of choice for papulopustular acne, even though the emerging resistant bacterial strains are minimizing their effect, especially regarding erythromycin. Systemic antibiotics should be administered during a period of 8–12 weeks. In severe papulopustular and in nodulocystic/conglobate acne, oral isotretinoin is the treatment of choice. Hormonal treatment represents an alternative regimen in female acne, whereas it is mandatory in resistant, severe pubertal or post-adolescent forms of the disease. Compounds with anti-androgenic properties include estrogens combined with progestins, such as ethinyl estradiol with cyproterone acetate, chlormadinone acetate, desogestrel, drospirenone, levonorgestrel, norethindrone acetate, norgestimate, and other anti-androgens directly blocking the androgen receptor (flutamide) or inhibiting androgen activity at various levels, corticosteroids, spironolactone, cimetidine, and ketoconazole. After 3 months of treatment control of

seborrhea and acne can be obtained. Low-dose corticosteroids (prednisone, prednisolone, or dexamethasone) are indicated in patients with adrenal hyperandrogenism or acne fulminans. New developments and future trends represent low-dose long-term isotretinoin regimens, new isotretinoin formulations (micronized isotretinoin), isotretinoin metabolites, combination treatments to reduce toxicity, insulin-sensitizing agents, 5 α -reductase type 1 inhibitors, antisense oligonucleotide molecules, and, especially, new anti-inflammatory agents, such as lipoxigenase inhibitors.

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Acne is a disorder of the pilosebaceous units located on the face, chest and back. It is an almost universal disease, occurring in all races, predominantly among adolescents [1–4]. Epidemiological studies have shown that about 70–87% of the adolescents experience acne lesions [5, 6]. The disease exhibits a peak incidence at 15–18 years of age. Spontaneous regression occurs in the majority of the patients after puberty, but in 10% of them acne persists over the age of 25 years and can last up to the 4th decade of life, and even up to the 6th decade of life in some cases.

As many as 15–30% of patients with acne need medical treatment because of the severity and/or persistence of their disease. In the years 1996–1998, more than 6 million visits per year to office-based physicians with acne as the principal reason have been registered in the USA; the

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Table 1. Simplified acne severity grading for the therapeutic decision

Severity grade	Comedones	Papules/pustules	Small nodules, cysts, fistules	Nodules	Inflammation	Scar formation
Mild	few	Ø or few	Ø	Ø	Ø	Ø
Moderate	numerous	few to many	Ø or few	Ø	marked	Ø
Severe	numerous	very numerous	many	Ø or few	strong	present
Very severe	fistule-comedones	very numerous	many	few to many, deeply located	very strong	present

patients received 6.5 million new prescriptions per year for systemic anti-acne drugs (antibiotics or isotretinoin) with a total cost likely to exceed USD 1 billion [7]. The different age ranges and the varying clinical pictures require better knowledge of the pathogenesis of the disease and clinical experience for its treatment [1–5, 8], especially since misconceptions regarding factors that exacerbate acne vulgaris not only exist in the community but have also been registered in last year medical students in an Australian study [9]. Several factors contribute to the pathogenesis of acne, among them increased sebaceous gland activity with hyperseborrhea [10], abnormal follicular differentiation and increased cornification [11], bacterial hypercolonization [12] as well as inflammation and immunological host reaction [13] are considered to be the major ones. Each of these factors provides a potential target for treatment. Genetic investigations have provided ambiguous proof for hereditary factors [14]; irregularities of the menstrual cycle, pregnancy, etc., have some influence on the acne course in females, and nutritional factors are accused to modify acne in some patients. Weather including ultraviolet light and other environmental factors may occasionally play a role. Several drugs can induce acne or acneiform lesions [3]. Psychological factors and stress have still no proven influence on the pathogenesis of acne but are often involved in its course. Recently, neuropeptides were reported to regulate the activity of the pilosebaceous unit [15, 16]. At last, acneiform eruptions can complicate the diagnosis.

Treatment of Acne: General Considerations

The exact classification and grading of acne is a fundamental requirement for the decision of the therapeutic regimen [1–4, 17–19]. In addition, acne at puberty needs subsequent prophylactic medication and care over several years after clinical healing. Infantile and pediatric acne,

androgenization signs in female patients with acne tarda [20, 21] or patients with signs of acne inversa may necessitate an alternative treatment. The compliance of the patient is an additional important parameter for the therapeutic strategy to be considered and its success. Skin type (dark skin tends to postinflammatory hyperpigmentation) and, especially, the tendency for scar formation play a role in the selection of treatment [22]. Two to 7% of the patients with acne experience a severe course associated with considerable scarring. A severe course associated with the presence of potential generators of physical and psychotic scars may require a therapeutic regimen based on systemic drugs [3, 18] (table 1).

Therapeutic Targets and Acne Drugs

Several clinical observations point to the importance of androgens in acne [23]. Androgens play an essential role in stimulating sebum production; androgen-insensitive subjects who lack functional androgen receptors do not produce sebum and do not develop acne. Moreover, systemic administration of testosterone and dehydroepiandrosterone increases the size and secretion of sebaceous glands [24–27]. Sebosuppression, i.e. suppression of sebaceous gland hyperactivity, can classically be achieved by systemic administration of anti-androgens or isotretinoin [19, 24–26, 28, 29] (table 2).

Abnormal keratinization of the infundibulum and the distal part of the sebaceous duct can be directly influenced through topical and systemic retinoids as well as through topical application of azelaic acid [30]. A number of further drugs can also secondarily induce keratolysis over their influence on other pathogenic factors [31]. Benzoyl peroxide and topical and systemic antibiotics primarily exhibit antimicrobial, but also anti-inflammatory activities [32, 33]. Various agents administered in acne treatment exhibit direct or indirect anti-inflammatory activi-

Table 2. Different action profile of systemic anti-acne drugs on the four major pathogenic factors of acne

	Follicular hyperkeratosis	Seborrhea	Bacterial hypercolonization	Inflammation
Antiandrogens(s)	—	++	—	—
Isotretinoin(s)	++	+++	(+)	++
Tetracyclines(s)	—	—	++	+

+++ = Very strong, ++ = strong, + = moderate, — = indirect/weak.

ties in addition to their effects on further pathogenic factors of acne. However, solely anti-inflammatory agents have rarely been administered [13].

Bacterial hypercolonization is not involved at the onset of acne, but it plays a role in the maintenance of the disease [2, 3, 8]. *Propionibacterium acnes* (*P. acnes*), an anaerobic bacterium, is a normal constituent of the cutaneous flora; however, it is virtually absent in the skin before puberty. Sebaceous follicles turning to microcomedones provide an anaerobic, lipid-rich environment for optimum bacterial proliferation. *P. acnes* produces lipases which can split triglycerides into free fatty acids. The latter can irritate the follicular cells and may cause hyperproliferation and/or inflammation. Topical or systemic antibiotics administered successfully in acne patients exhibit a suppressive effect on *P. acnes* proliferation but also directly suppress inflammation by decreasing neutrophil chemotaxis and down-regulating the expression of pro-inflammatory mediators and the production of chemotactic factors [34]. The unique environment of the pilosebaceous follicle makes lipophilic compounds clinically more active than hydrophilic ones [35].

Inflammation in acne has been considered as secondary to bacterial hypercolonization and, consequently, neither has it been carefully investigated nor become the target of treatment. The major hypothesis was that early during development of acne lesions neutrophils accumulate around and in the follicles through chemoattractive substances which may originate from *P. acnes* [1–4]. Hydrolytic enzymes and reactive oxygen species released by neutrophils promote tissue damage, facilitating the occurrence of debris within the lumen. The latter is considered to trigger the inflammatory cascade [36]. This hypothesis has gained support because several anti-acne drugs have been shown to inhibit the generation or activity of chemotactic factors or the release of reactive oxygen species [37]. In addition, linoleic acid, which is deficient in acne comedones, inhibits neutrophil oxygen metabolism and phagocytosis.

Table 3. Indications for oral antibiotic therapy in acne

Patients with moderate to severe acne
Patients for whom topical antibiotic therapy has failed or cannot be tolerated
Patients with moderate acne with tendency for scarring or substantial post-inflammatory hyperpigmentation
Patients with involvement of the shoulders, back or chest (difficult for topical application)

Systemic Treatment

Oral Antibiotics

Oral antibiotics are indicated for several groups of patients with inflammatory acne (table 3) [33, 38]. They include tetracyclines (tetracyclines, doxycycline, minocycline), erythromycin, clindamycin, and cotrimoxazole (table 4). These agents improve inflammatory acne by inhibiting the growth of *P. acnes*; tetracyclines and erythromycin have additional anti-inflammatory properties.

Tetracyclines of the first generation (tetracycline, oxytetracycline and tetracycline chloride) are the most commonly prescribed oral antibiotics for acne. They are used as a first-line agent because of their efficacy and low cost, although they have generated high rates of bacterial resistance. A 6-week treatment decreases the number of inflammatory lesions by approximately 50%. They are usually administered at a dose of 1 g/day (500 mg twice daily) over several months and after marked clinical improvement the dose can be reduced to 500 mg/day. Because their absorption is inhibited in the presence of food and dairy products, the drug must be taken preferably on an empty stomach one hour before meals with water for an optimal absorption.

Alternatively, tetracyclines of the second generation, namely doxycycline (initial dose of 100–200 mg/day with

Fig. 1. Mild acne papulopustulosa in a 24-year-old male patient before (left) and after a 6-month treatment with doxycycline 2 × 100 mg/day and topical tretinoin 0.1% (right).

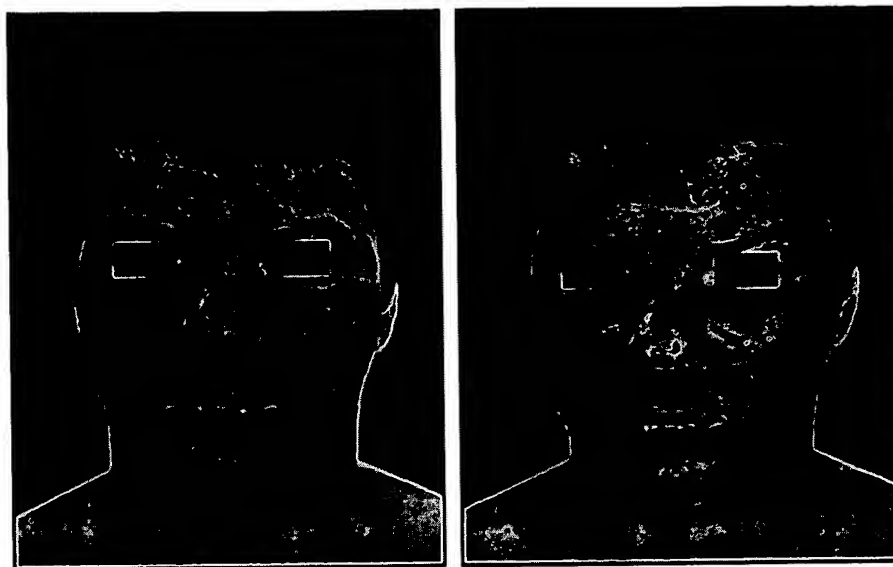


Table 4. Oral antibiotics used in acne treatment

Antibiotic	Usual dose	Comments
Tetracycline	250–500 mg × 2/day	low cost decreased absorption in presence of foods and dairy products
Doxycycline	100 mg × 2/day	may be taken with meals
Minocycline	50–100 mg × 2/day	expensive may be taken with meals safety problems
Erythromycin	500 mg × 2/day	common emergence of resistant <i>P. acnes</i> strains
Clindamycin	300 mg × 3/day	safety problems after long-term use
Cotrimoxazole	160/800 mg × 2/day	second-line therapy in acne

50 mg/day as maintenance dose) (fig. 1) and minocycline (usually 100 mg/day; 50 mg twice daily or 100 mg once daily) are more expensive but also more lipid soluble and better absorbed from the gastrointestinal tract. In contrast to tetracyclines of the first generation their absorption is not significantly limited by food, therefore, they can be taken with meals even though it is more effective when taken 30 min previously. Among tetracyclines, minocycline seems to induce more rapid clinical improvement as well as greater and more persistent reduction of inflammatory lesions and facial *P. acnes* counts, probably because it is the most lipophilic and may become highly concentrated in the pilosebaceous unit after its oral adminis-

tration [39]. Its major limitation occurs from currently observed significant safety problems (table 5) [40–43].

Erythromycin at a dosage of 1 g/day can be administered as an alternative regimen. It is equally effective with tetracycline; however, it induces higher rates of resistant *P. acnes* strains and may, therefore, be more often associated with treatment failures [12]. Its intolerable gastrointestinal side effects can be minimized by using intestine-soluble preparations.

Clindamycin is very effective but has disadvantages for long-term therapy because of the possible induction of pseudomembranous colitis. Cotrimoxazole (trimethoprim/ sulfamethoxazole, 160 mg/800 mg twice daily) is

effective in acne, however, it is recommended to reserve this drug for patients who responded inadequately to other antibiotics and for patients with gram-negative folliculitis.

Bacterial resistance is not rare after systemic administration of antibiotics over several months (table 5). Gastrointestinal upset under tetracycline and doxycycline with nausea, vomiting and diarrhea and vaginal candidosis under tetracycline are probably caused through changes in the gastrointestinal flora. Ultraviolet light sensitivity under tetracycline and doxycycline, not under minocycline, is frequent. Painful onycholysis has been occasionally observed under tetracycline treatment. Minocycline may cause allergic skin reaction, reversible vestibular disturbances (e.g. dizziness, vertigo, ataxia) and a blue-grey discoloration of the skin, particularly in inflamed areas, due to a reaction with free iron. Rarely, hepatitis and reactions resembling serum sickness and lupus erythematosus have been reported in association with oral use of tetracyclines, particularly minocycline. The teeth discoloration reported in children under 10 years can rarely also occur in adults. Tetracyclines are also accused for inducing benign intracranial hypertension which is, however, a rare adverse event. Tetracyclines must not be combined with systemic retinoids because the probability for development of intracranial hypertension increases. Since tetracyclines are contraindicated in pregnancy, erythromycin has to be administered as an alternative drug. Erythromycin causes the most frequent emergence of resistant *P. acnes* strains. It is also responsible for intolerable gastrointestinal side effects in many patients. Clindamycin treatment of acne is almost abandoned in several countries because of its association with pseudomembranous colitis due to intestinal colonization with *Clostridium difficile*. Metronidazole is then indicated in those cases. Appearance or enhancement of a vaginal candidosis can be observed in females, which frequently settles over the intestinal region.

Treatment with oral antibiotics should be administered for no less than 2 months but also generally not exceed 4–6 months [44]. Maximum clinical improvement is to be expected in the first 3–4 months; lack of improvement may indicate emergence of bacterial resistance [12]. Systemic antibiotics can be well combined with topical preparations, especially tretinoin, azelaic acid and benzoyl peroxide [45, 46].

Oral Isotretinoin

Oral isotretinoin is the most effective sebosuppressive agent and has revolutionized the treatment of severe acne

Table 5. Adverse events of systemic antibiotics

Adverse event	Compound
Bacterial resistance	tetracyclines > erythromycin > cotrimoxazole > minocycline
Gastrointestinal discomfort	clindamycin, tetracyclines
Pseudomembranous colitis	clindamycin
Postinflam. hyperpigmentation	minocycline > tetracycline
Vestibular disturbances	minocycline
Hypersensitivity reaction	minocycline, cotrimoxazole
Lupus erythematosus-like syndrome	minocycline
Interstitial nephritis/hepatic failure/systemic eosinophilia	minocycline

[28, 47–50]. It is the only drug currently available that affects all four pathogenic factors of acne. Like other retinoids, isotretinoin reduces comedogenesis. Moreover, it reduces sebaceous gland size (up to 90%) by decreasing proliferation of basal sebocytes, it suppresses sebum production in vivo and inhibits terminal sebocyte differentiation. Its stereoisomers tretinoin and alitretinoin (9-*cis* retinoic acid) were found inferior to isotretinoin in sebum suppression or acne treatment. Although not directly affecting *P. acnes*, its inhibitory effect on sebum production leads to alteration of the follicular microclimate and indirect fall of *P. acnes* counts reducing its ability to cause inflammation [51].

There is still debate as to the choice of dose. Some authors favor isotretinoin 0.5 mg/kg/day, others advocate higher dosage of 1 mg/kg/day. Although both regimens result to the same degree of long-term clinical improvement, relapse necessitating re-treatment occurs significantly more frequently under low-doses among patients with severe acne [52–53]. A 6-month treatment course is sufficient for 99% of the patients, but it has been documented that an initial dosage of 1 mg/kg/day for 3 months, then reduced to 0.5 and, if possible, to 0.2 mg/kg/day for 3–9 additional months will optimize the therapeutic outcome. As a rule, after 2–4 weeks of treatment, a 50% reduction of the pustules can be expected. Improvement continues during the post-treatment period. Relapses may occur after a single 6-month course. A 22–30% relapse rate was noted in patients followed for 10 years after having received isotretinoin 1 mg/kg/day (or cumulative dose ≥ 120 mg/kg), as compared to 39–82% with lower dose schedules [48].

Today, a 6- to 12-month course isotretinoin 0.5–1 mg/kg/day in most cases with severe acne, to reach a

Fig. 2. Severe acne papulopustulosa in a 21-year-old male patient before (left) and after a 4-month treatment with isotretinoin 0.5 mg/kg/day (right).

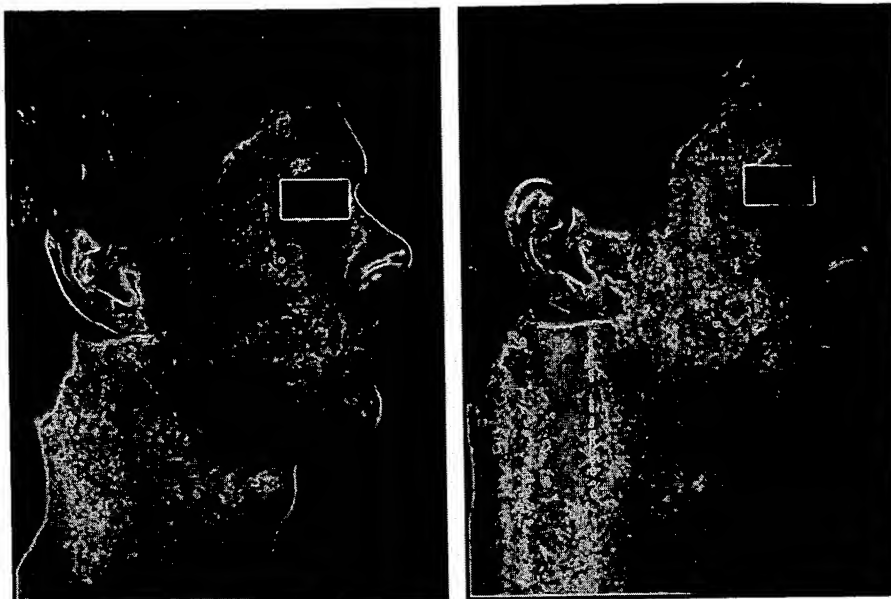
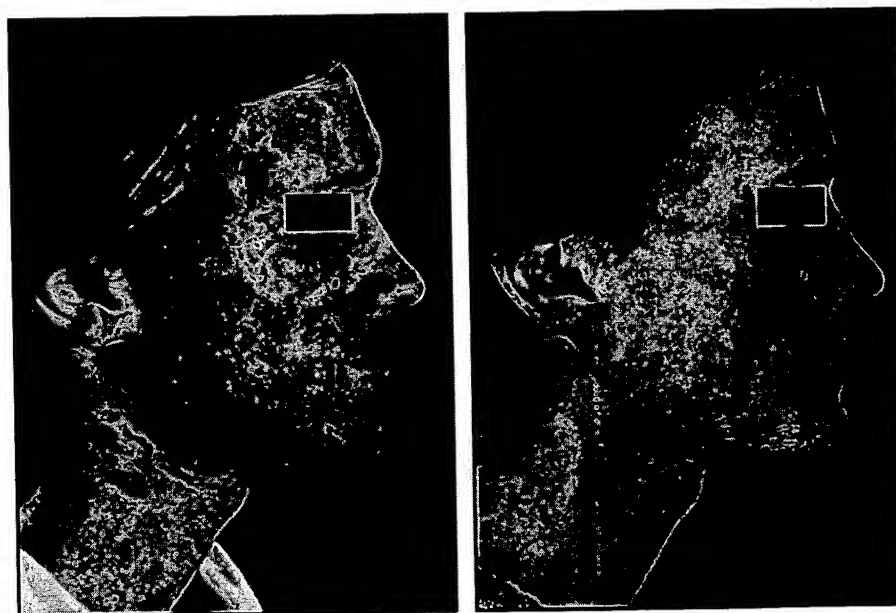


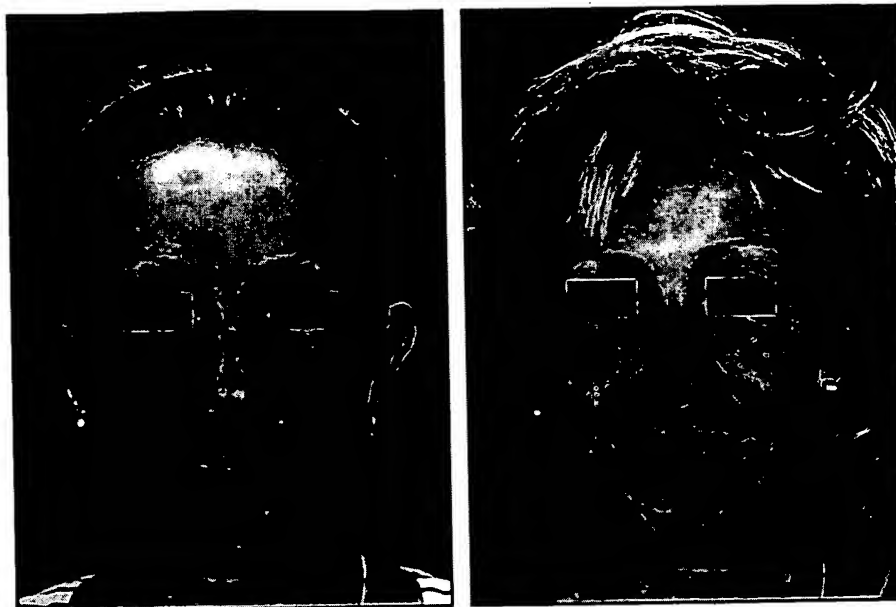
Fig. 3. Acne conglobata in an 18-year-old male patient before (left) and after a 6-month treatment with isotretinoin 1 mg/kg/day (cumulative dose 144 mg/kg) (right) [from ref. 28].



≥ 150 mg/kg total cumulative dose is recommended [28] (fig. 2–4). Three to 4 weeks after administration of the drug, an apparent flare-up may occur with increased development of inflammatory lesions which usually do not require modification of the oral dose and improve spontaneously. Factors contributing to the need for longer treatment schedules include low dose regimens (0.1–

0.5 mg/kg/day), presence of severe acne, extra-facial involvement and prolonged history of the disease. Higher dosages are indicated particularly for severe involvement of the chest and back [54]. Individual risk factors must be taken into account for establishing the dosage. Indications for optimal use are shown in table 6.

Fig. 4. Acne tarda without hormonal disturbances in a 44-year-old female patient before (left) and after a 12-month treatment with isotretinoin 0.5 mg/kg/day combined with ethinyl estradiol 35 µg/day – cyproterone acetate 2 mg/day (right).



The clinical course of isotretinoin therapy shows more rapid improvement of inflammatory lesions as compared to comedones. Pustules are cleared earlier than papules or nodules, and lesions localized on the face, upper arms and legs tend to clear more rapidly than trunk lesions [55]. Non-acne patients who have received oral isotretinoin therapy for seborrhea do not usually experience relapse for months or years. However, the duration of the sebostatic effect seems to be dose-dependent. Taking good tolerance into account, a dosage of 0.1–0.3 mg/kg/day over 4 weeks is sufficient to produce a sebostatic effect for at least 8 weeks after discontinuation of treatment. Five to 10 mg/day may be sufficient as a maintenance sebosuppressive dose over several years.

In female patients contraception is required and has to be enforced by the physician, because of the strong teratogenicity of isotretinoin [56, 57]. Isotretinoin can be well combined with a contraceptive pill which includes a hormonal anti-androgen [28, 57].

The adverse effect profile of oral isotretinoin is closely associated with hypervitaminosis A [28]. It includes a characteristic dose-dependent symptomatology with mucocutaneous side effects (table 7), elevation of serum lipids (approx. 20%), hyperostosis and extra-skeletal calcification (table 9). Arthralgia and myalgia may occur in up to 5% of individuals receiving high-dose isotretinoin. The major toxicity of isotretinoin results, however, from its

Table 6. Indications for optimal use of systemic isotretinoin

Severe acne (nodulocystica, conglobata, fulminans)
Patients with active acne and severe acne scars or potentially possible induction of physical or psychological scars
Patients with acne papulopustulosa who despite several conventional therapies, do not improve
Patients with acne papulopustulosa whose acne has responded well to conventional oral treatment on two or three occasions but has relapsed quickly after interruption of oral medication
Depressive and dysmorphic patients
In combination with oral contraceptive treatment in women with acne and signs of peripheral hyperandrogenism
Patients with excessive seborrhea
Patients with gram-negative folliculitis

teratogenic potential associated with high rate of spontaneous abortions and life-threatening congenital malformations. Therefore, the preparation can only be administered in women in combination with a secure contraceptive treatment or technique. Contraception is urgently recommended from 1 month before therapy, during the entire period of treatment and up to 3 months after discontinuation of the regimen. Oral isotretinoin treatment appears today strictly contraindicated in pregnancy, the

lactation period and in severe hepatic and renal dysfunction. Hyperlipidemia, diabetes mellitus and severe osteoporosis are relative contraindications. Co-medication with vitamin A (increased toxicity), tetracyclines (cranial hypertension) and high doses of aspirin (potentiation of mucosal damage) should be avoided. Liver and fat values in blood must be regularly controlled [58].

In long-term therapy (over 1–2 years), changes in the bone system with hyperostosis, periostosis, demineralization, thinning of the bones and premature calcification of epiphyses in adolescents have to be taken into consideration [59]. A radiograph and growth measurements are

reasonable tests before treatment of adolescents. Long-term adverse events after discontinuation of isotretinoin are rare.

Anti-Androgens

Hormonal anti-androgenic treatment can be administered in female patients to target the pilosebaceous unit and may inhibit sebum production by 12.5–65% (table 8) [25–27, 60, 61]. Once the decision has been made to initiate hormonal therapy, there are various options to choose among androgen receptor blockers and inhibitors of androgen synthesis at the levels of the ovary or the adrenal gland. Hormonal anti-androgenic treatment for acne must be continued for a sufficient period of time, at least 12 months and frequently longer. It is absolutely contraindicated in women who want to become pregnant due to the risk for sexual organ malformation in a developing fetus.

A most effective compound is cyproterone acetate, which belongs to the group of hydroxy-progesterones and blocks the binding of androgens to their receptors. There is current evidence that cyproterone acetate exhibits a dual activity by also inhibiting the synthesis of adrenal androgens because it inhibits the conversion of dehydroepiandrosterone to androstenedione by 3β -hydroxysteroid dehydrogenase/ Δ^5 -4-isomerase, which mainly occurs in the adrenal gland, and in the skin, in the sebaceous gland. Cyproterone acetate is incorporated in a marketed hormonal contraceptive at a dose of 2 mg in combination with 35 μ g ethinyl estradiol to avoid menstrual cycle problems [62–65] (fig. 5). The preparation can be used for both contraception and treatment of acne with or without signs of hyperandrogenism, even when serum androgen levels are normal. It has been shown to decrease serum gonadotropin, testosterone and androstenedione, with control of seborrhea and acne after three months treatment. In women with abnormal androgen metabolism additional cyproterone acetate 10–20 mg/day, and in some cases up to 50 mg/day can be administered orally during the first

Table 7. Mucocutaneous adverse events of isotretinoin (% values)

Cheilitis	75–95
Dermatitis facialis	30–50
Xerosis	30–50
Dry mucosa	20–50
Conjunctivitis	30
Epistaxis	25
Itching	25
Epidermal atrophy	15–25
Skin fragility	15–20
Desquamation	10–20
Hair loss	10–20
Retinoid dermatitis	5

Table 8. Indications for optimal use of hormonal therapy in women

Acne accompanied by mild or moderate hirsutism
Inadequate response to other acne treatments
Acne that began or worsened in adulthood
Premenstrual flares of acne
Excessive facial oiliness
Inflammatory acne limited to the 'beard area'

Table 9. Adverse events of systemic anti-acne drugs

Agent	Adverse event
Isotretinoin	teratogenicity, skin and mucosal dryness, irritation, bone changes, increase of the blood values for neutral lipids (cholesterol, triglycerides)
Hormonal contraceptives	edemas, thrombosis, increased appetite, weight gain, breast tenderness, decreased libido
Spironolactone	breast tenderness, menstrual irregularities, increased potassium blood levels

Fig. 5. Acne nodulocystica in a 20-year-old female patient before (left) and after a 6-month treatment with ethinyl estradiol 35 µg/day – cyproterone acetate 2 mg/day (right).



Fig. 6. Acne tarda with increased serum dihydrocpiandrosterone in a 31-year-old female patient before (left) and after a 2-month treatment with prednisolone 5 mg/day (right).



10 days of the menstrual cycle. Alternatively, a single i.m. injection of 100–300 mg cyproterone acetate can be applied at the beginning of the cycle.

There are other hormonal blockers of androgen receptors available, such as the gestagene chlormadinone acetate (2 mg) alone or in combination with 50 µg ethinyl estradiol or 50 µg mestranol in a contraceptive pill [66].

Most oral contraceptives contain two agents, estrogen (generally ethinyl estradiol) and a progestin. In their early formulations, oral contraceptives included high estrogen concentrations of over 100 µg which could directly suppress sebum production; low estrogen levels used currently act in the liver to increase the synthesis of sex hormone-binding globulin (SHBG). Circulating free testosterone

levels are reduced by the increased SHBG levels, leading to a decrease in sebum production. Oral contraceptives inhibit the ovarian production of androgens by suppressing ovulation. This, in turn, decreases serum androgen levels and reduces sebum production. On the other hand, the progestins administered belong to the families of estranes and gonanes with a variety of drugs in each class. Some progestins can cross react with the androgen receptor or, like the progestins norgestrel and levonorgestrel, reduce SHBG increasing free testosterone, thus leading to increased androgenic effects and aggravating acne, hirsutism, or androgenic alopecia [67, 68]. They can also cause changes in lipid metabolism and can increase serum glucose, leading to glucose intolerance, as well as possibly interfering with the beneficial effect of estrogen on the SHBG. Hormonal contraceptives are associated with edema, thrombosis, increased appetite, weight gain, breast tenderness and decreased libido [61].

Spironolactone, a synthetic steroid primarily acting as aldosterone antagonist, also blocks the androgen receptor exhibiting sufficient sebosuppression at doses 50–200 mg/day, a 2 × 25 mg regimen daily or at 4–22 days of cycle being the mostly used in anti-acne therapy. It may induce, however, cycle disturbances which can be corrected by non-androgenic progestins [69]. Spironolactone may induce dose-dependent breast tenderness, menstrual irregularities and increased potassium blood levels [70].

Flutamide, a synthetic compound which has mainly been administered to hirsute females, has been also shown to be active in acne after 1–6 months of treatment at doses of 250–500 mg/day (optimum 2 × 250 mg/day over 6 months) [63]. The agent becomes active through first-pass metabolism to 2-hydroxyflutamide. It inhibits binding of 5 α -dihydrotestosterone to its receptor protein and nuclear translocation of the receptor. Also, it may accelerate conversion of active androgens to inactive metabolites. Hepatic function laboratory tests should be done periodically [71].

Among nonhormonal anti-androgens, ketoconazole (cytochrome P-450 inhibitor and steroidogenesis enzyme blocker) in a dose of >200 mg/day and cimetidine (H₂-receptor antagonist) 5 × 300 mg/day exhibit weak anti-androgenic activity [70].

Gonadotropin-releasing agonists, such as buserelin, nafarelin or leuprolide, have been used to interrupt androgen production by the adrenals and ovaries by blocking FSH and LH liberation by the pituitary gland. These drugs are efficacious in acne and hirsutism, and are available as injectable drugs or nasal spray [25, 67]. However, in addition to suppressing the production of ovarian

androgens, they also suppress the production of estrogens, thereby eliminating the function of the ovary. Thus, the patient could develop menopausal symptoms and suffer from hypoestrogenism. They have variable acceptance due to the development of headaches as well as the occurrence of bone loss, due to the reduction in estrogen. They have not been registered for the treatment of acne.

Severe Inflammatory Acne and Acne fulminans

Systemic corticosteroids can become necessary in acne fulminans to suppress the excessive immunological reaction [54], in severe inflammatory forms of acne, and in order to prevent or treat a severe flare of the disease in the first 4 weeks of isotretinoin treatment. It is preferable to administer the corticosteroids for 3–4 weeks before administration of isotretinoin [72] but a combination of isotretinoin 0.5–1 mg/kg body weight/d and prednisolone 30 mg/day for 4–6 weeks (or other doses) with gradual reduction can also accelerate the conversion of fulminate disease course to common inflammatory acne [54, 73].

In contrast, oral non-steroidal anti-inflammatory agents have rarely been administered in the treatment of severe inflammatory acne forms.

Acne tarda

Systemic corticosteroids inhibit adrenal androgen liberation and, therefore, they are indicated in acne patients with adrenal hyperandrogenism and increased dihydroepiandrosterone levels, such as female patients with acne tarda [74]. This variant of acne tarda is characterized by inflammatory lesions, since increased dihydroepiandrosterone induces inflammation [75]. They are used at low prednisone, prednisolone (2.5–7.5 mg/day prednisolone) or dexamethasone doses [20] (fig. 6).

New Developments and Future Trends

After decades of stagnation, research on systemic acne treatment has expanded markedly in the last several years. The results of numerous studies have greatly increased our understanding of both the pathophysiology of the disease and the mechanisms of action for current therapies. New developments occurred including the low-dose long-term isotretinoin regimen, new isotretinoin formulations, understanding of isotretinoin's anti-sebotropic action, new antibiotics, and combination treatments to reduce toxicity and bacterial resistance, and new oral contraceptives. Future trends represent new anti-inflammatory agents, such as 5-lipoxygenase inhibitors, insulin-sen-

sitizing agents, 5 α -reductase type 1 inhibitors, and anti-sense molecules.

Low-Dose Isotretinoin

Low-dose isotretinoin (0.1–0.3 mg/ml/day daily or intermittent use) can effectively control acne, also being cost-effective. Nevertheless, the daily dose is too low for the cumulative dose obtained to be definitively curative. Although studies have been centered on the use of low doses only in older patients with exceptionally oily skin or in patients with long duration acne [76–80], there is a trend by practicing dermatologists to use low-dose isotretinoin in adolescent acne with a tendency to become inflammatory or in moderate acne as replacement of systemic antibiotics. The suggested rationale of such use is the effective control of inflammation with the final objective of preventing inflammation and the resulting scars. The approach taken is that of control and not of absolute resolution, since this resolution will occur in the majority of patients naturally. The simultaneous use of an effective topical therapy is mandatory. Since a large percentage of patients to be treated with mini-doses are women, they should be made to understand that the teratogenesis risk is the same as with the complete dose. Adverse events with these low doses are almost absent.

New Isotretinoin Formulations

A recent study by Strauss et al. [81] using a micronized isotretinoin formulation with higher bioavailability exhibited similar efficacy results of a single daily 0.4 mg/kg dose of micronized isotretinoin and 1.0 mg/kg standard isotretinoin administered in two divided doses after 20 weeks of treatment. Micronized isotretinoin presented a safety profile similar to that of standard isotretinoin with a lower risk of mucocutaneous adverse events and hypertriglyceridemia [82].

Understanding the Unique Activity of Isotretinoin

The high anti-sebotropic activity of isotretinoin is particularly surprising because of the fact that it has low binding affinities for both cellular retinoic acid-binding proteins I and II as well as for nuclear retinoic acid receptors [83, 84]. Because retinoids are thought to exert most of their effects by modulating gene expression and/or activating nuclear retinoid receptors, it has been suggested that isotretinoin may act as a pro-drug that becomes active after isomerization to tretinoin acid or conversion to alitretinoin [84]. Indeed, current results reported by Tsukada et al. [85] have shown that isotretinoin undergoes significant isomerization to tretinoin in cultured

sebocytes, an effect being specific for these cells. In addition, administration of isotretinoin to sebocytes only led to a delayed induction of the cytochrome P450 isoenzymes responsible for tretinoin inactivation. Isotretinoin effects were found to be dependent on the extra-cellular albumin concentration [86]. On the other hand, tretinoin acted via retinoic acid receptors (RAR) to exert its anti-proliferative effect on sebocytes. Therefore, the molecular basis for this anti-sebotrophic activity is probably a selective intracellular isomerization of isotretinoin to tretinoin in human sebocytes, with isotretinoin representing a pro-drug for tretinoin in this specific tissue. Newer data indicate that isotretinoin metabolites, such as 4-oxo-isotretinoin, may also represent compounds exhibiting direct anti-acne activity.

In addition to the better understanding of isotretinoin activity, new possible adverse events have emerged. The proposed relationship between the compound and depression as well as suicide was reviewed not to be based on a putative molecular mechanism of the compound indicating that there is no evidence to support a casual connection [87]. On the other hand, 38 different signs and symptoms of ocular abnormalities were reported as 'certain' to have resulted from the use of isotretinoin, among them decreased dark adaptation may jeopardize adolescents under the drug who drive in the night [88].

New Antibiotics

Limecycline is a second-generation tetracycline linked to the amino acid lysine, with an efficacy similar to that of doxycycline and minocycline [89]. It is used at a 300 mg initial dose that is lowered to 150 mg after 2 weeks. It exhibits excellent tolerance with scarce risk of hyperpigmentation, vestibular disorders and photosensitivity, and can be administered together with food.

Roxithromycin, a macrolide antibiotic, is administered in a dose of 150 mg twice daily in the treatment of inflammatory acne. It accumulates at therapeutic levels in the pilosebaceous system [90] and exhibits an interesting spectrum of effects, namely direct anti-inflammatory and anti-androgenic activities. It significantly inhibits the production of lipase and neutrophil chemotactic factor by *P. acnes* as well as of *P. acnes*-induced NF- κ B activation at concentrations much lower than the MIC at which the growth curve of *P. acnes* is not affected [34, 91]. In addition, roxithromycin was found to serve as anti-androgen only in the hypersensitive state to androgens, but not in the physiological state through modulating end-organ hypersensitive condition to androgens [92].

Azithromycin, another macrolide antibiotic, was found as effective as doxycycline (100 mg/day) administered in a dose of 500 mg once a day for 4 days per month for a total of 12 weeks on a pure protocol basis and statistically significantly better than doxycycline by intention to treat analysis [93].

In an open study, levofloxacin was found effective for inflammatory acne and achieved high levels in the lesions [94].

Combination Treatments

Combinations of a topical retinoid (adapalene, tretinoin) or azelaic acid with oral antibacterial agents are recommended to induce maximum anti-inflammatory effect in mild to moderate inflammatory acne [45, 46, 93, 95]. Such combinations can lead to a rapid dose reduction and quicker discontinuation of oral antibiotics increasing the effectiveness, improving the compliance, and reducing the development of bacterial resistance to antibiotics.

New Oral Contraceptives

When oral contraceptives are administered in the treatment of acne, it is possible that some women are more sensitive to the androgenic effects of a progestin, but it is more likely that the effect of progestin may be offset by the estrogen. Although some progestins might be more androgenic than others, all oral contraceptives, regardless of the type of progestin each contains, increase SHBG and inhibit serum androgen levels. This is also possible with the marketed combination of ethinyl estradiol (20 µg) and levonorgestrel (100 µg; one of the older and most androgenic progestins) found to produce a significant decrease in comedones, as well as in papules and pustules [66, 96, 97].

The concentrations of estrogen in oral contraceptives have decreased over the years from 150 to 35 µg, and in the most recent forms to 20 µg, in order to reduce the side effects of estrogen. On the other hand, many progestins have been developed over the years and the third-generation progestins, including desogestrel, drospirenone, gestodene, and norgestimate, are more selective for the progesterone receptor rather than the androgen receptor. The combinations of ethinyl estradiol (30–40 mg) and desogestrel (25–125 µg) [65, 98], ethinyl estradiol (20–35 µg) and norethindrone acetate (1 g) [99], ethinyl estradiol (30 mg) and drospirenone (3 mg) [64], and ethinyl estradiol and norgestimate (180–250 µg) [100, 101] have been marketed as contraceptive pills; among them those including norethindrone acetate and norgestimate have been approved for acne [27].

New Anti-Inflammatory Agents

It is widely accepted that inflammation in acne vulgaris may be mainly induced by an immunologic reaction to extracellular products of *P. acnes* [102]. However, it is by no means clear that either bacteria or their products initiate follicular inflammation. Ingham et al. [103] investigated the presence of pro-inflammatory cytokines in open acne comedones from untreated acne patients and found bioactive interleukin(IL)-1 α -like material. The majority of open comedones also contained micro-organisms, but there was no significant correlation between levels of any cytokine, in particular IL-1 α , and numbers of micro-organisms.

Additional results have shown that the sebaceous gland expresses a number of different cytokines at steady state, without the influence of any external factors. Antilla et al. [104] showed that IL-1 is present in normal sebaceous glands and Boehm et al. [105] used in situ hybridization techniques to show that messenger RNA (mRNA) for IL-1 α , IL-1 β and tumor necrosis factor- α is present at multiple sites in normal skin including the sebaceous glands. Thus, while the presence of bacteria, most notably *P. acnes*, may stimulate upregulation of cytokine expression in sebaceous glands [106], pro-inflammatory cytokines are expressed in these tissues in the absence of defined external influences.

Guy et al. [107] assessed the action of IL-1 α in the microdissected human pilosebaceous infundibulum preparations in vitro and found an IL-1 α -specific induction of hypercornification of the infundibulum similar to that seen in comedones. Follicular keratinocytes and sebocytes in vitro were also found to produce pro-inflammatory cytokines and chemokines [108]. Currently, inflammation has been suggested to occur due to enhancement of IL-8 production in human monocytes and sebocytes through a mechanism requiring transcription factor NF- κ B activation [34, 108] and involvement of Toll-like receptor 2 [109, 110]. These results provide logical support for the use of anti-inflammatory regimens in the treatment of acne [13].

The use of anti-inflammatory drugs for the treatment of acne is further supported by recent results indicating a key role for leukotriene B₄ (LTB₄) in the development of tissue inflammation [111]. LTB₄ is a pro-inflammatory mediator synthesized from arachidonic acid. Synthesis of LTB₄ is catalyzed by 5-lipoxygenase and leukotriene A₄ hydrolase and is increased by inflammatory mediators including endotoxin, complement fragments, tumor necrosis factor- α and interleukins. LTB₄ induces recruitment and activation of neutrophils, monocytes and eosin-

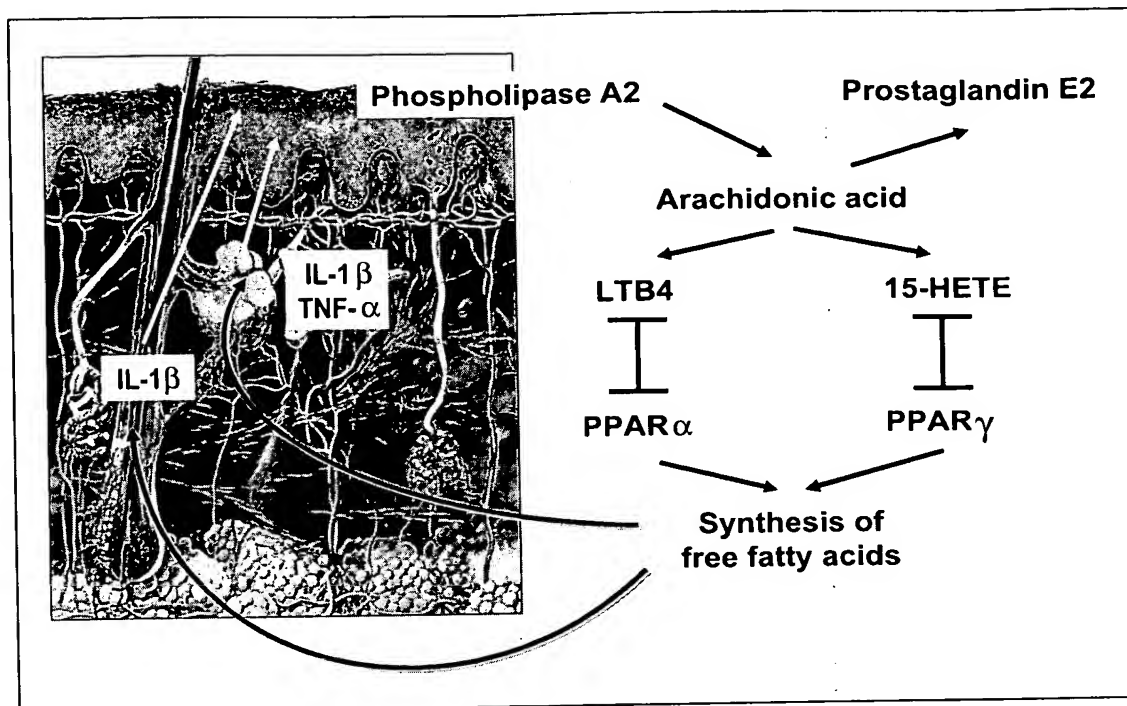


Fig. 7. The cascade of eicosanoid synthesis in the skin, as inflammatory signaling pathway possibly involved in the development of acne lesions. IL-1 β = Interleukin-1 β ; TNF- α = tumor necrosis factor- α ; LTB4 = leukotriene B4; 15-HETE = 15-hydroxyeicosatetraenoic acid; PPAR = peroxisome proliferator-activated receptor [from ref. 24].

ophils. It also stimulates the production of a number of pro-inflammatory cytokines and mediators that augment and prolong tissue inflammation (fig. 7). Limited data from pharmacological inhibition studies support a role for LTB4 in the pathogenesis of neutrophil-mediated tissue damage.

The potential importance of this inflammatory pathway for acne treatment was evaluated in a small cohort of patients [112]. A 3-month study of the effectiveness of a specific lipoxygenase inhibitor was performed by systemic administration in 10 patients with inflammatory acne. Clinical evaluation of these patients indicated an approximately 60% decrease in the acne severity index within 3 weeks of the initiation of treatment and a 70% reduction in inflammatory lesions at 3 months. Additional evaluation indicated an approximately 65% reduction in total sebum lipids as well as a substantial decrease in lipoperoxides. Free fatty acids were also decreased by almost 80%. Bivariate analysis indicated that the decrease in total sebum lipids, and especially in pro-inflammatory lipids, was directly correlated with the improvement in

inflammatory lesions. Thus, the results of this small-scale clinical trial and associated laboratory analysis strongly support the conclusion that appropriate anti-inflammatory therapy has the potential to effectively treat acne. These results also support the view that sebum lipids induce inflammation in acne, independent of the presence of bacteria or increased systemic levels of pro-inflammatory molecules.

Eleven years ago, Wozel et al. [113] assessed the ability of isotretinoin as well as a number of other agents to inhibit transdermal migration of polymorphonuclear leukocytes stimulated by LTB4. Topical treatment with isotretinoin resulted in a marked and statistically significant inhibition of the LTB4-induced migration of polymorphonuclear leukocytes. Retinoids are nowadays considered to regulate inflammation [114, 115] probably also using the Toll-like receptor 2 pathway [116].

Insulin-Sensitizing Agents

Since insulin has a direct effect on ovarian androgen production in vitro, insulin resistance may play a crucial

role in the physiopathology of peripheral hyperandrogenism, including acne [117]. Insulin-sensitizing agents have recently been investigated for their role in the short term treatment of insulin resistance in polycystic ovary syndrome. Controlled studies have shown that metformin administration, by promoting body weight loss, can decrease fasting and stimulated plasma insulin levels. However, other studies have shown metformin 500 mg 3 × daily to decrease insulin secretion and to reduce ovarian production of 17 α -hydroxyprogesterone with recovery of spontaneous or clomifene-induced ovulation, independently of weight loss. These findings suggest a new indication for metformin and present insulin-sensitizing agents as a novel approach in the treatment of ovarian hyperandrogenism.

Peroxisomes play an important role in regulating cellular proliferation and differentiation as well as in the modulation of inflammatory mediators. In addition, peroxisomes have broad effects on the metabolism of lipids, hormones, and xenobiotics [118]. On the other hand, activation of peroxisome proliferator-activated receptor (PPAR)- γ and - α by their respective specific ligands, thiazolidinedione and clofibrates, was found to induce lipid droplet formation in rat preputial gland cells (resembling sebocytes) but not epidermal cells in vitro [119]. PPAR- γ 1 mRNA was also demonstrated in rat preputial gland cells but not in epidermal cells. These findings are compatible with the concepts that PPAR- γ 1 gene expression plays a unique role in the differentiation of sebocyte-like cells. These findings have implications for the development of new modalities of treatment for acne vulgaris and explain why lipoxygenase inhibitors inhibit lipid synthesis [112]: The lipoxygenase products LTB₄ and 15-HETE are natural ligands of PPAR- α and PPAR- γ , respectively.

5 α -Reductase Type 1 Inhibitors

The inhibitors of 5 α -reductase isoenzymes (1 and 2) can be schematically divided in three groups according to their substrate specificity: Pure or preferential inhibitors of 5 α -reductase 1, pure or preferential inhibitors of 5 α -reductase 2, and dual inhibitors [26, 120]. Despite the fact that several steroidal and non-steroidal inhibitors have been synthesized and experimented in pharmacological models, only finasteride has been extensively used for clinical purposes, namely benign prostate hyperplasia and male baldness with positive results. In women, finasteride has been used in some control trials for treatment of hirsutism with an objective favorable response. On the basis of experimental observations on distribution of 1 and 2 isoenzymes in human skin, scalp and prostate, the pure 5 α -

reductase 1 inhibitors seem the ideal drugs for treatment of acne and hirsutism [121–123] and have been introduced in clinical studies [27].

Antisense Molecules

The androgen receptor is involved in the development of acne and its expression can classically be regulated by androgen receptor blockers. A more elegant way is the transient transfection of skin cells with antisense oligonucleotides against the androgen receptor [124]. The development of thioat- and ribosyl-antisense oligonucleotides against the androgen receptor led with high specificity in a transient diminished protein expression of the receptor and to a strong inhibition of the biological activity of androgens in human sebocytes and keratinocytes in vitro. Such experiments are only in an initial phase. The future clinical use of such highly specific compounds is dependent on several factors, among them being the effective administration pathway and the kind of transfection systems to be applied.

Conclusion

Despite the interest on the development of topical treatments for acne in the last decades [30], systemic treatment is still a milestone, especially in the treatment of moderate-to-severe scarring types of the disease. The establishment of new systemic drugs for acne is based on the consideration of successes and pitfalls of the past and the emerging knowledge of the future [125]. Among all pathogenetic factors of acne, inflammation seems to be rediscovered [13] and anti-inflammatory concepts seem to become the new trend of systemic and topical acne treatment.

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